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(54) Title: RADIOLABELING KIT AND BINDING ASSAY

(57) Abstract

Antibody binding assays and radiolabeling kits are disclosed for radiolabeling and testing therapeutic antibodies in the commercial setting. In particular, the kits are designed for making and evaluating radiolabeled anti-CD20 conjugates to be used for the treatment and imaging of B cell lymphoma tumors. All kit reagents are sterile and are designed to achieve a high level of antibody radiolabeling and product stability with results which are highly reproducible.

RADIOLABELING KIT AND BINDING ASSAY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to antibody binding assays and radiolabeling kits, lyophilized cell preparations, reagents and protocols for testing the clinical efficacy of therapeutic antibodies for the treatment/imaging of tumors and tumor cells. Specifically, the kits of the present invention are used for making and evaluating radiolabeled antibody conjugates that will be used for the treatment and imaging of B-cell lymphoma tumors by targeting the B cell surface antigen BP35 ("CD20").

2. Technology Background

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The immune system of vertebrates (for example, primates, which include humans, apes, monkeys, etc.) consists of a number of organs and cell types which have evolved to: accurately and specifically recognize foreign microorganisms ("antigen") which invade the vertebrate-host; specifically bind to such foreign microorganisms; and, eliminate/destroy such foreign microorganisms. Lymphocytes, as well as other types of cells, are critical to the immune system. Lymphocytes are produced in the thymus, spleen and bone marrow (adult) and represent about 30% of the total white blood cells present in the circulatory system of humans (adult).

There are two major sub-populations of lymphocytes: T cells and B cells. T cells are responsible for cell mediated immunity, while B cells are responsible for antibody production (humoral immunity). However, T cells and B cells can be considered as interdependent – in a typical immune response, T cells are activated when the T cell receptor binds to fragments of an antigen that are bound to major histocompatability complex ("MHC") glycoproteins on the surface of an antigen presenting cell; such activation causes release of biological mediators ("interleukins") which, in essence, stimulate B cells to differentiate and produce antibody (immunoglobulins") against the antigen.

Each B cell within the host expresses a different antibody on its surface--thus one B cell will express antibody specific for one antigen, while another B cell will express antibody specific for a different antigen. Accordingly, B cells are quite diverse, and this diversity is critical to the immune system. In humans, each B cell can produce an enormous number of antibody molecules (i.e., about 10^7 to 10^8). Such antibody production most typically ceases (or substantially decreases) when the foreign antigen has been neutralized. Occasionally, however, proliferation of a particular B cell will continue unabated; such proliferation can result in a cancer referred to as "B cell lymphoma."

T cells and B cells both comprise cell surface proteins which can be utilized as "markers" for differentiation and identification. One such human B cell marker is the human B lymphocyte-restricted differentiation antigen Bp35, referred to as "CD20." CD20 is expressed during early pre-B cell development and remains until plasma cell differentiation. Specifically, the CD20 molecule may regulate a step in the activation process which is required for cell cycle initiation and differentiation and is usually expressed at very high levels on neoplastic ("tumor") B cells. CD20, by definition, is present on both "normal" B cells as well as "malignant" B cells, i.e., those B cells whose unabated proliferation can lead to B cell lymphoma. Thus, the CD20 surface antigen has the potential of serving as a candidate for "targeting" of B cell lymphomas.

In essence, such targeting can be generalized as follows: antibodies specific to the CD20 surface antigen of B cells are, e.g., injected into a patient. These anti-CD20 antibodies specifically bind to the CD20 cell surface antigen of (ostensibly) both normal and malignant B cells; the anti-CD20 antibody bound to the CD20 surface antigen may lead to the destruction and depletion of neoplastic B cells. Additionally, chemical agents or radioactive labels having the potential to destroy the tumor can be conjugated to the anti-CD20 antibody such that the agent is specifically "delivered" to, e.g., the neoplastic B cells. Irrespective of the approach, a primary goal is to destroy the tumor: the specific approach can be determined by the particular anti-CD20 antibody which is utilized and, thus, the available approaches to targeting the CD20 antigen can vary considerably.

For example, attempts at such targeting of CD20 surface antigen have been reported. Murine (mouse) monoclonal antibody 1F5 (an anti-CD20 antibody) was reportedly administered by continuous intravenous infusion to B cell lymphoma patients. Extremely high levels (>2 grams) of 1F5 were reportedly required to deplete circulating tumor cells, and the results were described as being "transient." Press et al., "Monoclonal Antibody 1F5 (Anti-CD20) Serotherapy of Human B-Cell Lymphomas," Blood 69/2:584-591 (1987).

A potential problem with this approach is that non-human monoclonal antibodies (e.g., murine monoclonal antibodies) typically lack human effector functionality, i.e., they are unable to, inter alia, mediate complement dependent lysis or lyse human target cells through antibody dependent cellular toxicity or Fc-receptor mediated phagocytosis. Furthermore, non-human monoclonal antibodies can be recognized by the human host as a foreign protein; therefore, repeated injections of such foreign antibodies can lead to the induction of immune responses leading to harmful hypersensitivity reactions. For murine-based monoclonal antibodies, this is often referred to as a Human Anti-Mouse Antibody response, or "HAMA" response. Additionally, these "foreign" antibodies can be attacked by

the immune system of the host such that they are, in effect, neutralized before they reach their target site.

Lymphocytes and lymphoma cells are inherently sensitive to radiotherapy. Therefore, B cell malignancies are attractive targets for radioimmunotherapy (RIT) for several reasons: the local emission of ionizing radiation of radiolabeled antibodies may kill cells with or without the target antigen (e.g., CD20) in close proximity to antibody bound to the antigen; penetrating radiation, i.e., beta emitters, may obviate the problem of limited access to the antibody in bulky or poly vascularized tumors; and, the total amount of antibody required may be reduced. The radionuclide emits radioactive particles which can damage cellular DNA to the point where the cellular repair mechanisms are unable to allow the cell to continue living; therefore, if the target cells are tumors, the radioactive label beneficially kills the tumor cells. Radiolabeled antibodies, by definition, include the use of a radioactive substance which may require the need for precautions for both the patient (i.e., possible bone marrow transplantation) as well as the health care provider (i.e., the need to exercise a high degree of caution when working with radioactivity).

Therefore, an approach at improving the ability of murine monoclonal antibodies to effect the treatment of B-cell disorders has been to conjugate a radioactive label to the antibody such that the label or toxin is localized at the tumor site. Toxins (i.e., chemotherapeutic agents such as doxorubicin or mitomycin C) have also been conjugated to antibodies. *See*, for example, PCT published application WO 92/07466 (published May 14, 1992).

"Chimeric" antibodies, i.e., antibodies which comprise portions from two or more different species (e.g., mouse and human) have been developed as an alternative to "conjugated" antibodies. Mouse/human chimeric antibodies have been created, and shown to exhibit the binding characteristics of the parental mouse antibody, and effector functions associated with the human constant region. *See* e.g., Cabilly et al., U.S. Patent 4,816,567; Shoemaker et al., U.S. Patent

4,978,745; Beavers et al., U.S. Patent 4,975,369; and Boss et al., U.S. Patent 4,816,397 all of which are incorporated by reference herein. Generally these chimeric antibodies are constructed by preparing a genomic gene library from DNA extracted from pre-existing murine hybridomas. Nishimura et al. (1987) Cancer Research 47: 999. The library is then screened for variable region genes from both heavy and light chains exhibiting the correct antibody fragment rearrangement patterns. The cloned variable region genes are then ligated into an expression vector containing cloned cassettes of the appropriate heavy or light chain human constant region gene. The chimeric genes are then expressed in a cell line of choice, usually a murine myeloma line.

For example, Liu, A.Y., et al., "Production of a Mouse-Human Chimeric Monoclonal Antibody to CD20 with Potent Fc-Dependent Biologic Activity", J. Immun. 139/10:3521-3526 (1987), describes a mouse/human chimeric antibody directed against the CD20 antigen. *See* also, PCT Publication No. WO 88/04936. However, no information is provided as to the ability, efficacy or practicality of using Liu's chimeric antibodies for the treatment of B cell disorders in the reference.

It is noted that *in vitro* functional assays (e.g. complement dependent lysis ("CDC"); antibody dependent cellular cytotoxicity ("ADCC"), etc.) cannot inherently predict the *in vivo* capability of any antibody to destroy or deplete target cells expressing the specific antigen. *See*, for example, Robinson, R.D., et al., "Chimeric mouse-human anti-carcinoma antibodies that mediate different anti-tumor cell biological activities," *Hum. Antibod. Hybridomas*, 2:84-93 (1991) (chimeric mouse-human antibody having undetectable ADCC activity). Therefore, the potential therapeutic efficacy of antibodies can only truly be assessed by *in vivo* experimentation.

To this end, copending applications 08/475,813, 08/475,815 and 08/478,967, herein incorporated by reference in their entirety, disclose radiolabeled anti-CD20 conjugates for diagnostic "imaging" of B cell lymphoma

tumors before administration of therapeutic antibody. "In2B8" conjugate comprises a murine monoclonal antibody, 2B8, specific to human CD20 antigen, that is attached to Indium[111] (^{111}In) via a bifunctional chelator, i.e., MX-DTPA (diethylenetriaminepentaacetic acid), which comprises a 1:1 mixture of 1-isothiocyanatobenzyl-3-methyl-DTPA and 1-methyl-3-isothiocyanatobenzyl-DTPA. Indium-[111] is selected as a diagnostic radionuclide because it emits gamma radiation and finds prior usage as an imaging agent.

Patents relating to chelators and chelator conjugates are known in the art. For instance, U.S. Patent No. 4,831,175 of Gansow is directed to polysubstituted diethylenetriaminepentaacetic acid chelates and protein conjugates containing the same, and methods for their preparation. U.S. Patent Nos. 5,099,069, 5,246,692, 5,286,850, and 5,124,471 of Gansow also relate to polysubstituted DTPA chelates. These patents are incorporated herein in their entirety.

The specific bifunctional chelator used to facilitate chelation in applications 08/475,813, 08/475,815 and 08/478,967 was selected as it possesses high affinity for trivalent metals, and provides for increased tumor-to-non-tumor ratios, decreased bone uptake, and greater *in vivo* retention of radionuclide at target sites, i.e., B-cell lymphoma tumor sites. However, other bifunctional chelators are known in the art and may also be beneficial in tumor therapy.

Also disclosed in applications 08/475,813, 08/475,815 and 08/478,967 are radiolabeled therapeutic antibodies for the targeting and destruction of B cell lymphomas and tumor cells. In particular, the Y2B8 conjugate comprises the same anti-human CD20 murine monoclonal antibody, 2B8, attached to yttrium-[90] (^{90}Y) via the same bifunctional chelator. This radionuclide was selected for therapy for several reasons. The 64 hour half-life of ^{90}Y is long enough to allow antibody accumulation by the tumor and, unlike e.g. ^{131}I, it is a pure beta emitter of high energy with no accompanying gamma irradiation in its decay, with a range of 100 to 1000 cell diameters. The minimal amount of penetrating radiation allows for outpatient administration of ^{90}Y-labeled antibodies. Furthermore, internalization of

labeled antibodies is not required for cell killing, and the local emission of ionizing radiation should be lethal for adjacent tumor cells lacking the target antigen.

Because the ^{90}Y radionuclide was attached to the 2B8 antibody using the same bifunctional chelator molecule MX-DTPA, the Y2B8 conjugate possesses the same advantages discussed above, e.g., increased retention of radionuclide at a target site (tumor). However, unlike ^{111}In, it cannot be used for imaging purposes due to the lack of gamma radiation associated therewith. Thus, a diagnostic "imaging" radionuclide, such as ^{111}In, can be used for determining the location and relative size of a tumor prior to and/or following administration of therapeutic chimeric or ^{90}Y-labeled antibodies for the purpose of tumor reduction. Additionally, indium-labeled antibody enables dosimetric assessment to be made.

Depending on the intended use of the antibody, i.e., as a diagnostic or therapeutic reagent, other radiolabels are known in the art and have been used for similar purposes. For instance, radionuclides which have been used in clinical diagnosis include ^{131}I, ^{125}I, ^{123}I, ^{99}Tc, ^{67}Ga, as well as ^{111}In. Antibodies have also been labeled with a variety of radionuclides for potential use in targeted immunotherapy (Peirersz et al. (1987) The use of monoclonal antibody conjugates for the diagnosis and treatment of cancer. Immunol. Cell Biol. 65: 111-125). These radionuclides include ^{188}Re and ^{186}Re as well as ^{90}Y, and to a lesser extent ^{199}Au and ^{67}Cu. I-[131] has also been used for therapeutic purposes. U.S. Patent No. 5,460,785 provides a listing of such radioisotopes and is herein incorparted by reference.

As reported in copending applications 08/475,813, 08/475,815 and 08/478,967 administration of the radiolabeled Y2B8 conjugate, as well as unlabeled chimeric anti-CD20 antibody, resulted in significant tumor reduction in mice harboring a B cell lymphoblastic tumor. Moreover, human clinical trials reported therein showed significant B cell depletion in lymphoma patients infused with chimeric anti-CD20 antibody. In fact, chimeric 2B8 has recently been heralded the nation's first FDA-approved anti-cancer monoclonal antibody under

the name of Rituxan®. Thus, at least one chimeric anti-CD20 antibody has been shown to demonstrate therapeutic efficacy in the treatment of B cell lymphoma.

In addition, U.S. Application Serial No. 08/475,813, herein incorporated by reference, discloses sequential administration of Rituxan®, a chimeric anti-CD20, with both or either indium-labeled or yttrium-labeled murine monoclonal antibody. Although the radiolabeled antibodies used in these combined therapies are murine antibodies, initial treatment with chimeric anti-CD20 sufficiently depletes the B cell population such that the HAMA response is decreased, thereby facilitating a combined therapeutic and diagnostic regimen.

Thus, in this context of combined immunotherapy, murine antibodies may find particular utility as diagnostic reagents. Moreover, it was shown in U.S. Application 08/475,813 that a therapeutically effective dosage of the yttrium-labeled anti-CD20 antibody following administration of Rituxan® is sufficient to (a) clear any remaining peripheral blood B cells not cleared by the chimeric anti-CD20 antibody; (b) begin B cell depletion from lymph nodes; or (c) begin B cell depletion from other tissues.

Thus, conjugation of radiolabels to cancer therapeutic antibodies provides a valuable clinical tool which may be used to assess the potential therapeutic efficacy of such antibodies, create diagnostic reagents to monitor the progress of treatment, and devise additional therapeutic reagents which may be used to enhance the initial tumor-killing potential of the chimeric antibody. Given the proven efficacy of an anti-CD20 antibody in the treatment of non-Hodgkin's lymphoma, and the known sensitivity of lymphocytes to radioactivity, it would be highly advantageous for such therapeutic antibodies to become commercially available in kit form whereby they may be readily modified with a radiolabel and administered directly to the patient in the clinical setting.

Although there exist many methods and reagents for accomplishing radiolabeling of antibodies, what is lacking in the art is a convenient vehicle for placing these reagents in the clinical setting, in a way that they may be easily

produced and administered to the patient before significant decay of the radiolabel or significant destruction of the antibody due to the radiolabel occurs. The lack of such convenient means to commercialize this valuable technology could be due to the poor incorporation efficiencies demonstrated by some known labeling protocols, and the subsequent need to column purify the reagent following the radiolabeling procedure. The delay in development of such kits might also in part be due to the previously lack of accessibility to pure commercial radioisotopes which may be used to generate efficiently labeled products absent subsequent purification. Alternatively, perhaps the reason such kits are generally unavailable is the actual lack of antibodies which have been able to achieve either the approval or the efficacy that Rituxan® has achieved for the treatment of lymphoma in human patients.

For instance, as discussed in U.S. Patent 4,636,380, herein incorporated by reference, it has been generally believed in the scientific community that for a radiopharmaceutical to find clinical utility, it must endure a long and tedious separation and purification process. Indeed, injecting unbound radiolabel into the patient would not be desirable. The need for additional purification steps renders the process of radiolabeling antibodies in the clinical setting an impossibility, particularly for doctors who have neither the equipment nor the time to purify their own therapeutics.

Furthermore, radiolabeled proteins may be inherently unstable, particularly those labeled with radiolytic isotopes such as ^{90}Y, which have the tendency to cause damage to the antibody the longer they are attached to it in close proximity. In turn, such radiolysis causes unreliable efficiency of the therapeutic due to loss of radiolabel and/or reduced binding to the target antigen, and may lead to undesired immune responses directed at denatured protein. Yet without the facilities for labeling and purifying the antibodies on site, clinicians have had no choice but to order therapeutic antibodies already labeled, or have them labeled off site at a related facility and transported in following labeling for administration to

the patient. All such manipulations add precious time to the period between labeling and administration, thereby contributing to the instability of the therapeutic, while in effect decreasing the utility of radiolabeling kits in the clinical setting.

Others have tried unsuccessfully to develop antibody radiolabeling kits that would be proficient enough to forego a separate purification step of the antibody. For instance, Cytogen has recently launched a commercial kit for radiolabeling a murine monoclonal antibody directed to tumor-associated glycoprotein TAG-72. However, Cytogen's antibody is particularly unamenable to a kit formulation due to the tendency to develop particulates during storage which must later be removed by a further filtration step. Moreover, Cytogen's antibody has caused adverse reactions in patients due to a HAMA responses.

Others have claimed to have developed radiolabeling protocols which would be amenable to kit format in that a separate purification step would not be required (Richardson et al. (1987) Optimization and batch production of DTPA-labeled antibody kits for routine use in ^{111}In immunoscintography. Nuc. Med. Commun. 8: 347-356; Chinol and Hnatowich (1987) Generator-produced yttrium-[90] for radioimmunotherapy. J. Nucl. Med. 28(9): 1465-1470). However, such protocols were not able to achieve the level of incorporation that the present inventors have achieved using the protocols disclosed herein, which have resulted in incorporation efficiencies of at least 95%. Such a level of incorporation provides the added benefit of increased safety, in that virtually no unbound label will be injected into the patient as a result of low radioincorporation.

The protocols included in the kits of the present invention allow rapid labeling which may be affected in approximately a half an hour or as little as five minutes depending on the label. Moreover, the kit protocols of the present invention have a labeling efficiency of over 95% thereby foregoing the need for further purification. By foregoing the need for further purification, the half-life of

the radiolabel and the integrity of the antibody is reserved for the therapeutic purpose for which it is labeled.

The present application discloses convenient kits and methods whereby diagnostic and therapeutic antibodies may be radiolabeled and administered to a patient in a reproducible, reliable and convenient manner. The kits of the present invention transform the process of radiolabeling antibodies into a hassle-free, worry-free standardized process, which greatly facilitates patient treatment protocols. The present kits provide advantages over the prior art in that the optimum parameters for labeling and administering therapeutic or diagnostic have been determined, thereby reducing the cost of goods. Since the kits described herein provide the optimum parameters according to the particular label, use of a kit designed for a particular label will also minimize cannibalization, i.e., which occurs when an inappropriate kit is used for a particular label. Avoiding cannibalization in turn also provides for optimum labeling efficiency. Moreover, the protocols and sterile, pyrogen-free ingredients included with each kit make for a more user-friendly process, since sterility, pyrogen testing and post-labeling purification of the reagents are obviated.

3. Summary of the Invention

The present invention includes a kit for radiolabeling a diagnostic or therapeutic antibody before administration to a patient comprising at least (i) a vial containing a chelator-conjugated antibody, (ii) a vial containing formulation buffer for stabilizing and administering the radiolabeled antibody, and (iii) instructions for radiolabeling the antibody, wherein said vial components are supplied in such an amount and at such a concentration that when they are combined with a radiolabel of sufficient purity and activity according to the kit instructions, no further purification of the labeled antibody is required before administration to said patient. Moreover, when labeled according to the kit instructions and with a

radioisotope of sufficient purity and activity, such isotope incorporation may reach levels higher than 95%, and even as high as 98% or higher.

The antibody included in the kit is most preferably an anti-CD20 antibody. The antibody is supplied in a form whereby it is attached to a bifunctional chelator. Preferably, the antibody is conjugated to MX-DTPA, but other chelators such as phenyl- or benzyl-conjugated DTPA, cyclohexyl-DTPA, EDTA derivatives and DOTA may be used. A chelator according to the present invention may be any chelator that is at least bifunctional, i.e., which possesses at least two binding sites (at least one site for chelating a metallic ion and at least one site for coupling to a protein ligand).

Depending on the antibody used, the conjugated antibody is typically supplied at a concentration of 0.5 to 30 mg/ml, more preferably 2 mg/ml. The volume of conjugated antibody will depend on the concentration and the amount required for optimum labeling depending on the radiolabel. However, the conjugated antibody is to be supplied in such a volume and concentration that the entire volume will be added to the reaction vial using a sterile syringe and aseptic technique. This will allow for increased reproducibility and ease of use. All reagents of the kits disclosed herein are sterile and pyrogen-free, and specifically designed for simplicity and speed in advancing directly from antibody testing to administration. With some labels, the need for testing labeling efficiency may not be required.

A particularly advantageous component of the kit is the formulation buffer for stabilizing against the effects of radiolysis and administering the radiolabeled conjugated antibody to a patient. The formulation buffer is a pharmaceutically acceptable carrier which serves as both a diluent for the labeled antibody and an administration buffer. Although any pharmaceutically acceptable diluent may be used for administering therapeutic or diagnostic antibodies to patient, the formulation buffer of the present invention is particularly suited for administering radiolabeled antibodies.

For instance, the formulation buffer of the present invention comprises a radioprotectant such as human serum albumin (HSA) or ascorbate, which minimize radiolysis due to yttrium, and to a lesser degree, indium. Other radioprotectants are known in the art and could also be used in the formulation buffer of the present invention, i.e., free radical scavengers (phenol, sulfites, glutathione, cysteine, gentisic acid, nicotinic acid, ascorbyl palmitate, HOP(:O)H_2, glycerol, sodium formaldehyde sulfoxylate, $Na_2S_2O_5$, $Na_2S_2O_3$, and SO_2, etc.).

It should be noted that, while radioprotectants are generally employed in the formulation buffer to protect the antibody from radiolysis, it may be possible to affect further protection by including the radioprotectant in the reaction buffer as well. This has generally not been done before, i.e., with HSA, due to the presence of metals which would interfere with the labeling process. However, it may be possible to "clean" the HSA using a chelating resin such that it could be included in the reaction buffer as well. Ascorbate or other radioprotectants may also need to be treated to remove contaminating metals.

The formulation buffer of the present invention also comprises excess unconjugated chelator. The purpose for including unconjugated chelator is that this chelator serves to scavenge any non-protein bound radiolabel in the patient, and effects excretion of the radiolabel thereby reducing uptake of "bone-seeking" isotopes, i.e., ^{90}Y, by the bones of the patient. For instance, when the antibody of the kit is conjugated to a DTPA chelator, excess DTPA or any other chelator may be included in the formulation buffer. The formation buffer is also preferably supplied in a volume such that the entire contents are transferred to the reaction vial. As discussed above, this results in increased ease of use and reproducibility because exact volumes do not have to be measured and transferred.

A preferred formulation buffer comprises phosphate buffered or physiological saline, human serum albumin and DTPA. The human serum albumin is preferably at a concentration of between about 1 to 25% (w/v), and more preferably at a concentration of about 7.5% (w/v). The concentration of

DTPA is preferably about 1 mM. Ascorbate may be used as an alternative to human serum albumin, and is typically used at a concentration of about 1 to 100 mg/ml. Although a wider range of concentrations may be used without compromising patient safety.

The antibody of the radiolabeling kit is readily labeled with a radioisotope of choice via a bifunctional chelator according to the methods of the present invention. For further simplicity in this regard, the kit of the present invention may also include a vial containing a buffer for adjusting the pH of the radioisotope solution, and a sterile glass reaction vial for performing the labeling and subsequently for resuspending the final radiolabeled antibody in formulation buffer. A 10 ml reaction vial is typically sufficient, but vials capable of holding 5 to 20 mls may also be used. The buffer is preferably a low-metal sodium acetate solution at a concentration of 10 to 1000 mM, most preferably 50 mM.

A specific kit of the present invention comprises the MX-DTPA conjugated antibody, 2B8-MX-DTPA. 2B8 is an anti-CD20 antibody shown to affect B cell depletion upon administration to lymphoma patients. However, it should be apparent to those skilled in the art that the radiolabeling kit of the present invention may be optimized for the radiolabeling of other anti-CD20 antibodies, or any other antibody which has been conjugated to DTPA or other polyvalent chelator. The preferred kit of the present invention may comprise at least (i) a vial containing the MX-DTPA conjugated 2B8 antibody, either in solution or lyophilized (requiring reconstitution); and (ii) a vial containing formulation buffer for administering the radiolabeled antibody to a patient. The preferred kit will also contain (iii) a buffer for adjusting isotope pH, and (iv) a reaction vial. Alternatively, and more preferably, the buffer is supplied in the reaction vial, thereby eliminating the steps of measuring and transferring the buffer and increasing the simplicity, consistency and sterility of the kit components, However, other embodiments are also envisioned, i.e., whereby the buffer is added to the isotope vial first, and the buffered isotope is then transferred to the reaction vial. In this case, the reaction

vial could be supplied with the required antibody volume. Alternatively, the isotope/buffer vial could be made large enough to accommodate addition of the antibody conjugate, i.e., directly to the supplier's vial. This would eliminate the need for the reaction vial.

As described above, another preferred kit configuration is encompassed whereby the reaction vial itself contains the required volume of conjugated antibody (i.e., 1 or 1.5 mL for ^{111}In and ^{90}Y, respectively). The antibody may be supplied in a buffer that provides the appropriate radiolabeling pH according to the specific desired isotope (i.e., pH 3-6 for ^{111}In, pH 3-5 for ^{90}Y). Different buffers may be used, depending on the isotope (i.e., sodium acetate for ^{90}Y, sodium citrate for ^{111}In). The pH and composition of the buffer may also vary depending on the nature of the binding ligand to be labeled (i.e., labeling peptides may permit $<$ pH 3 to be used). Essentially then, the isotope would be transferred directly to the reaction vial, as would the formulation buffer. Limiting use of the kit to two transfer steps would further increase reproducibility and simplicity, and further decrease the chance for contamination of sterility during manipulation of the kit components.

The radiolabeling kits of the present invention may further comprise a vial of radioisotope, or radioisotope may be ordered separately from an appropriate supplier. Preferred radioisotopes of the present invention are ^{111}In chloride and ^{90}Y chloride in HCl although the disclosed methods are not limited to these isotopes. Other radionuclides that have been used for imaging applications are known in the art, i.e., as described in U.S. Patent Nos. 4,634,586, 5,460,785 and 5,766,571, which are herein incorporated by reference. Indium-[111] is particularly advantageous for imaging B cell tumors and beta emitters such as ^{90}Y are particularly useful as radiotherapeutic agents. Although other radioisotopes suitable for these or other purposes, i.e., alpha emitters, may be used depending on the chelator used for antibody conjugation.

Given the proven efficacy of the combined therapeutic regimens disclosed in U.S. Application Serial No. 08/475,813, a further kit embodiment will also include a separate vial of chimeric antibody, i.e., Rituxan®, to be administered before or after the radiolabeled anti-CD20 antibody. When the chimeric antibody is administered before the radiolabeled antibody, the HAMA response which might generally occur in response to administration of a murine anti-CD20 antibody may be significantly decreased, thereby increasing the therapeutic utility of radiolabeled murine antibodies. Moreover, when chimeric anti-CD20 is employed to clear circulating B cells, subsequent diagnostic images achieved with ^{111}In-labeled antibodies may be much clearer.

It should also be apparent that both a diagnostic radiolabeled antibody and a therapeutic radiolabeled antibody may be used together in a combined therapeutic regimen. In this regard, the diagnostic antibody may be used either before or after the therapeutic antibody to visualize tumor size before and after treatment. In this case, the kit of the present invention may include separate, perhaps color-coded, buffer vials specifically formulated according to the optimum pH requirements for radiolabeling antibodies with the specific radioisotopes to be used. Such a system would ensure that the appropriate buffer was used for each label, and would allow the clinician the same ease in radiolabeling the two antibodies as if two kits had been purchased. Such a kit in effect combines the components from two radiolabeling kits into one.

The components of the radiolabeling kit of the present invention are supplied at the appropriate concentration and pH so that sterility is readily maintained before antibody administration and there is little need for additional buffers or media. However, it should be apparent to those of skill in the art that some of the reagents can be prepared, sterilized and tested for sterility on site. Thus, variations of the kit of the invention are envisioned depending on the budget and preference of the consumer.

The radiolabeling kit of the present invention may be used in a method for radiolabeling a chelator-conjugated antibody for administration to a patient. According to the present invention, such a method comprises, in general, (i) mixing a chelator-conjugated antibody with a solution containing a radioisotope; (ii) incubating the mixture for an appropriate amount of time at appropriate temperature; and (iii) diluting the labeled antibody to an appropriate concentration in formulation buffer, such that the radiolabeled antibody may be administered directly to a patient without further purification.

Most preferably the antibody is an anti-CD20 antibody, and in particular, the anti-CD20 antibody may be 2B8. The antibody may be conjugated to any appropriate chelator, i.e., MX-DTPA, CHX-DTPA, phenyl- or benzyl-DTPA, DOTA, EDTA derivatives, etc. MX-DTPA is preferred. Methods for affecting antibody conjugation are known in the art (Kozak et al. (1989); Mirzadeh et al. (1990), Brachbiel et al. (1986)).

The present inventors have found that the method of radiolabeling a chelator-conjugated antibody works best wherein the solution containing the radiolabel is adjusted to a pH of between about 3.0 and 6.0, and more preferably to about 4.2 before it is mixed with the chelator-conjugated antibody. Low-metal sodium acetate is particularly preferred for adjusting the pH, although other buffers may be used. Preferably, the sodium acetate is at a concentration of between about 10 and 1000 mM, and more preferably 50 mM.

When the radioisotope is ^{111}In chloride, the volume quantity of ^{111}In chloride which should be used to prepare a single administrative dose is typically about 5.5 mCi divided by the radioactivity concentration at the time of labeling. For patient administration, a typical diagnostic dose of ^{111}In is about 2 to 10 mCi. The quantity of sodium acetate used for adjusting the pH varies depending on the sodium acetate concentration and the isotope carrier solution, and may therefor be quite broad. When the concentration of sodium acetate is 50 mM, the amount required for adjusting the pH is typically about 1.2 times the volume quantity of

^{111}In chloride used although larger volumes may be used. It should be appreciated that the ratio of sodium acetate to HCl is what is important, and the amount of sodium acetate used would change depending on the amount and concentration of HCl in the buffer. About 1 ml of a chelator-conjugated antibody at a concentration of about 2 mg/ml is then mixed with the radiolabel acetate solution, and the mixture is incubated for about 30 minutes, or for a time sufficient to achieve optimal labeling of the antibody. Such time may range from about 30 seconds to about 60 minutes. Formulation buffer is then added in an amount necessary to achieve a total final volume of about 10 ml.

The optimum time required for labeling the antibody may vary depending on the antibody, the particular radiolabel and the particular conjugate employed. An underlying factor in the optimization of the time allotted for radiolabeling is the chelator to antibody ratio of the reagent which is to be labeled. For instance, the chelator to antibody ratio must be high enough to achieve a therapeutically useful level of incorporation, i.e., 90 to 95% depending on the radioisotope, but must also not be too high such that the structural integrity or immunoreactivity of the antibody is compromised. This requires a certain balancing process that in some cases may lead to a lower level of conjugated chelator and longer labeling time.

For instance, for 2B8 and MX-DTPA, it has been discovered that labeling may be accomplished in under five minutes for ^{90}Y and in about thirty minutes for ^{111}In to achieve the desired level of radioincorporation, with only about a 1½ to 1 molar ratio of chelator to antibody. It was not necessary, therefor, to increase the chelator to antibody ratio, because a desirable level of radioincorporation was achieved. Moreover, it was not advantageous to increase the quantity of conjugated chelator because this could effect antibody immunoreactivity. Such parameters could be empirically determined for other antibodies for the design of kits such as those described in the present invention.

When the radioisotope is ^{90}Y chloride, the volume quantity of ^{90}Y chloride used for preparing a single administrative dose typically ranges from about 10 to

50 mCi, and is preferably about 45 mCi, divided by the radioactivity concentration at the time of labeling. The quantity of sodium acetate used for adjusting the pH varies depending on the sodium acetate concentration and the concentration of isotope carrier, and may therefor be quite broad. When the concentration of sodium acetate is 50 mM and the ^{90}Y is supplied in 50 mM HCl, the amount required for adjusting the pH is typically about 1.2 times the volume quantity of ^{90}Y chloride used. About 1.5 ml of a chelator-conjugated antibody at a concentration of about 2 mg/ml is then mixed with the radiolabel acetate solution, and incubated for about 5 minutes, or for a time sufficient to achieve optimal labeling of the antibody. Such time may range from about 30 seconds to about 60 minutes. Formulation buffer is added in an amount necessary to achieve a total final volume of about 10 ml.

Preferably, the radiolabeling method of the invention is performed using the radiolabeling kit described herein. However, it should be apparent to those of skill in the art that the preferred components and conditions are merely acceptable guidelines for practicing the method of the invention, and may be altered to some degree with appropriate optimization. Conditions which depart from those preferred but still accomplish the purpose of the method are considered to be within the scope of the invention.

The radiolabeling kit of the present invention may also be supplied with reagents suitable for conveniently verifying the binding affinity of the antibody following radiolabeling. In such a case, the kit of the invention may also be used for determining the percent binding of a radiolabeled antibody to its target cell before administering the antibody to a patient. The present inventors have also found that the particular binding assay kit disclosed may be useful for testing the affinity of any antibody generally for which purified antigen is not available. Accordingly, the binding assay components may also be sold as a separate kit.

In general, a binding assay and radiolabeling kit comprises (i) at least one vial of lyophilized cells which express the antigen which is recognized by the

antibody in the kit; (ii) a vial containing chelator-conjugated antibody; (iii) a vial containing formulation buffer, and (iv) instructions for radiolabeling the antibody such that the radiolabeled antibody may be administered directly to a patient without the need for subsequent purification. As described above for the radiolabeling kit, this kit may also comprise a vial containing a buffer for adjusting the pH of the radioisotope, and a sterile glass reaction vial. Preferably the buffer is a low-metal sodium acetate solution at a concentration of between about 10 and 1000 mM, and the glass reaction vial holds a volume of at least 5 ml. The antibody is preferably an anti-CD20 antibody, and the chelator is preferably MX-DTPA. Other chelators may be used as described previously. The preferred conjugated antibody is 2B8-MX-DTPA, although any chelator-conjugated antibody may be labeled and its affinity assessed. The formulation buffer is phosphate buffered saline comprising a radioprotectant and unconjugated chelator as described above, and radioisotope may or may not be included and is preferably ^{111}In chloride or ^{90}Y chloride. Other radioisotopes may be used depending on the chelator.

The difference between the binding assay/radiolabeling kit and the radiolabeling kit described above is the inclusion of antigen-positive cells to serve as a substrate target for testing antibody affinity. When the antigen is CD20, preferred CD20-positive cells are SB cells (ATCC # CCL 120) but any CD20-positive cells may be used. The binding assay and radiolabeling kit may further include antigen-negative cells for use as a negative control. Preferred CD20-negative cells are HSB cells (ATCC # CCL 120.1) but any CD20-negative cells may be used.

Of course, the combined radiolabeling and binding assay kit may further comprise a vial of chimeric anti-CD20 antibody in addition to the antibody to be labeled for the purposes of affecting a combined therapeutic regimen, or for clearing peripheral B cells prior to diagnostic imagery. Such separate antibody is preferably Rituxan®, but may be any antibody shown to effectuate tumor cell

killing. In fact, two different types of antibodies may be combined in one kit, i.e., antibodies directed to two different B cell antigens, so long as the combined therapeutic regimen serves to target the same type of cell, i.e., the B cell lymphoma.

Just as the components of the kit may be used to label other antibodies, other cells for testing antibody affinity may be prepared depending on the target antigen. However, for anti-CD20 antibodies, the binding assay and radiolabeling kit of the present invention is particularly suited for the commercial setting in that the target cells are provided in lyophilized form. This allows the verification of antibody efficacy to proceed simply and systematically, and negates the hassle and expense involved in maintaining tissue culture facilities. The lyophilized cells are generally supplied in aliquots of between 0.5 and 500 X 10^6 cells per vial according to the methods of the invention.

It is possible that particular facilities will prefer to order antibody which has already been radiolabeled, in which case such a facility might desire the binding assay reagents in order to ensure that the antibodies retain target affinity. In this case, the present invention also provides for a binding assay kit for determining the percent binding of a radiolabeled antibody to its target cell. Such a kit includes at least one vial of fixed and/or lyophilized antigen-positive cells, and may optionally contain antigen-negative cells as described above for the binding assay and radiolabeling kit. Moreover, it should be apparent that variations of such a kit may include an unlabeled control antibody for verifying the binding specificity of the consumer's antibody via a competitive assay.

Again, when the antigen is CD20, the CD20-positive cells are preferably SB cells (ATCC # CCL 120) and the CD20-negative cells are preferably HSB cells (ATCC # CCL120.1), which are supplied in lyophilized form in aliquots of between 0.5 and 50 X 10^6 cells. In this case, the antibody is preferably an MX-DTPA conjugate of 2B8 labeled with ^{111}In or ^{90}Y.

In view of the additional kit embodiments disclosed herein, it should be stressed that one of the advantages of the radiolabeling kit and method of the present invention is that no further purification step is necessary, and the radiolabeled antibody may be administered directly to the patient, thereby saving valuable time and increasing antibody stability. Therefor, it is emphasized that, while it might be desirable for the clinician to test or verify the binding specificity and affinity of the radiolabeled antibody prior to administration, such test may be foregone with particular radioisotopes if antibody stability and the inhibition of radiolysis are particular concerns, i.e., as with yttrium. By providing kit embodiments whereby the binding affinity and specificity may be tested, the present inventors are in no way suggesting that such tests are absolutely required in the methods or kits of the invention. The option to test such antibody validity is purely at the option of the clinician.

The present inventors have also found that the method used for preparing fixed and lyophilized cells for the binding assay kits of the present invention is particularly suitable for preparing cells for commercial kits. Cells may be fixed prior to lyophilization to improve structure/stability. In particular, the cells of the present invention demonstrate high reproducibility when used for antibody binding assays.

In particular, the present invention includes a method of preparing lyophilized cells comprising (i) harvesting cells at a cell density of 0.5 to 2 X 10^6 cells per ml by centrifugation; (ii) washing cells at least one time in a balanced salt solution, i.e., HBSS; (iii) resuspending pelleted cells in a lyophilization buffer comprising a balanced salt solution containing carrier protein and at least one type of sugar; (iv) dispensing an aliquot of resuspended cells into a microfuge tube or a glass vial; and (v) lyophilizing the cells 12-96h and more preferably 24-72h at about 30-60 millitor. The method is particularly suitable for preparing lyophilized cells wherein said cells are SB cells (ATCC # CCL 120) or HSB cells (ATCC # CCL 120.1), but is likely applicable to other cell types as well.

Preferably, the buffer generally contains bovine serum albumin as the carrier protein at a concentration of 1% (w/v) and mannitol at a concentration of 10%. However, conceivably other carrier proteins, i.e., HSA, and other sugars may be used. The cells are harvested by centrifugation at a speed of about 1300 rpm, and the salt solution HBSS (Hank's balanced salt solution) is added. The cells are generally resuspended at a concentration of 50 X 10^6 cells per ml. However, it should be apparent to those of skill in the art that the above conditions may be modified slightly without significantly compromising cell viability. Moreover, the above conditions may be supplemented by additional procedures designed to optimize the process for larger quantities of cells, e.g., tangential flow diafiltration to exchange cells into the lyophilization buffer.

The binding assay kits of the present invention may be used in an assay for assessing the binding affinity of a radiolabeled antibody. Such an assay is also a subject of the present invention. A binding assay for determining the percent binding of a radiolabeled antibody to its target cell comprises in general the following steps: (i) mixing at least one aliquot of a radiolabeled antibody with at least one aliquot of antigen positive cells; (ii) mixing at least one aliquot of a radiolabeled antibody identical to the aliquot of step (i) with at least one aliquot of dilution buffer of the same volume as the aliquot of antigen-positive cells in step (i) as a control; (iii) pelleting the cells by centrifugation; (iv) measuring the radioactivity in the supernatant of the pelleted cells and the control; and (v) comparing the quantity of radioactivity in the cell supernatant to the quantity of radioactivity in the control.

Just as the radiolabeling kits of the present invention optionally contain ^{111}In chloride or ^{90}Y chloride, the binding assay of the present invention is typically performed with antibodies labeled with ^{111}In or ^{90}Y. When ^{111}In is the radiolabel, radioactivity in the assay tubes is measure using a gamma counter. When ^{90}Y is the label, radioactivity is measured using a scintillation counter, although a gamma counter could be used.

For the binding assay of the present invention, the preferred antibody is an anti-CD20 antibody, and the anti-CD20 antibody is preferably 2B8, wherein the 2B8 antibody is labeled using the radiolabeling kit of the present invention. However, any radiolabeled antibody may be tested provided cells expressing the particular antigen are available. When CD20 is the antigen the preferred cells for performing the assay are SB cells (ATCC # CCL 120), however, the assay may also be optimized and performed with any radiolabeled antibody and appropriate target cell.

The dilution buffer used for the assay should maintain binding of the antibody, i.e., physiological buffer, possibly containing a carrier protein, e.g. BSA, to minimize non-specific binding to cells. Although the tube with dilution buffer serves as a control, a further control may be included in the assay by using antigen-negative cells. In this case, the binding assay further comprises the following steps: (i) mixing at least one aliquot of a radiolabeled antibody with at least one aliquot of antigen-negative cells; (ii) pelleting the antigen-negative cells by centrifugation; (iv) measuring the radioactivity in the supernatant of the antigen-negative pelleted cells; and (v) comparing the quantity of radioactivity in the antigen-negative cell supernatant to the quantity of radioactivity in the supernatant of the antigen-positive cell supernatant and the control. Comparing the radioactivity obtained from this tube to the dilution buffer control will serve as a measure of the amount of non-specific binding to antigen-positive cells. When CD20 is the antigen, and the CD20-positive cells are SB cells, CD20 negative cells are preferably HSB cells (ATCC # CCL 120.1).

As described above, the lyophilized cells of the present invention provide a simple, efficient and reproducible standard for testing the binding efficacy of a radiolabeled antibody. Therefore, the binding assay of the present invention is preferably performed using the lyophilized cells included in the binding assay kits of the present invention. In addition, the radiolabeling assays of the present invention may be combined with the binding assays of the present invention,

wherein the antibody is first labeled by the method of labeling an chelator-conjugated antibody as described in the present invention. Most preferably, the binding assay of the present invention is performed using one of the binding assay and radiolabeling kits described herein.

There may be some instances where the affinity of an antibody should be tested or verified but a radiolabel has not been attached. For instance, under certain circumstances, i.e., trouble-shooting, it may be advantageous to test the binding affinity of an antibody before radiolabeling. For such a case, the present invention also encompasses a competitive binding assay for assessing affinity of a test antibody to a target cell, comprising (i) preparing a ruthenium-labeled control antibody using a known antibody specific for the same antigen; (ii) incubating increasing amounts of test antibody and increasing amounts of unlabeled control antibody with a fixed concentration of target cells and a trace amount of ruthenium-labeled antibody wherein each separate concentration of test antibody and each separate concentration of control antibody are in separate tubes, respectively; (iii) determining the quantity of binding in each reaction tube based on relative electrochemiluminescence (ECL) using ORIGEN instrumentation; and (iv) calculating the average affinity value of the test antibody. The average affinity value may be calculated from the EC50 values and the known concentration of trace antibody using the method of Muller (J. Immunological Methods (1980) 34:345) or any other appropriate method. It should be noted that this assay may also be used to test the affinity of radiolabeled antibodies, or any antibody for which antigen cannot be purified and cells are required as an antigen source. The fixed, lyophilized cells of the present invention may be used as target cells.

When the competitive binding assay of the present invention is performed to test the affinity of anti-CD20 antibodies, the control antibody may be 2B8, or any other unconjugated anti-CD20 antibody. The control antibody may be a chelator-conjugated antibody. The test antibody may also be a chelator-conjugate of the control antibody. Alternatively, the test antibody may be another anti-CD20

antibody whose binding affinity to CD20 as compared to 2B8 is of interest. However, the assay may be adapted for use with antibodies having other specificities so long as an appropriate target cell is available.

In the competitive binding assay of the present invention, the preferred target cells are CD20-positive cells, more preferably SB cells (ATCC # CCL 120), and are more preferably resuspended lyophilized SB cells prepared according to the method of the present invention. Cells lyophilized using other methods or fixed cells may also be used. The ruthenium-labeled antibody is typically prepared by a process comprising incubating the control antibody with N-hydroxysuccinimide ester of ruthenium (II) tris-bypyridine chelator (TAG-NHS), although other known method of labeling antibodies are also envisioned. For labeling, the control antibody and TAG-NHS are preferably incubated at about a 1:15 molar ratio.

These and other aspects of the present invention will become clearly understood by reference to the following figures, examples and description of the invention.

4. Brief Description of the Drawings

Figure 1. Immunoreactivity of native 2B8 was compared to commercially available anti-CD20 antibodies B1 (Coulter) and Leu 16 (Becton Dickinson) by direct competition in a radioimmunoassay using ^{125}I-labeled B1. Antigen-positive SB cells (100,000) were added to each well of V&P filter plates; 10 ng of radiolabeled B1 was mixed with various concentrations of unlabeled competition and the mixture added to the cells. The antibodies were incubated with the cells for one hour at ambient temperature; determinations were performed in triplicate. Subsequently, the wells were washed, dried and the filter-associated radioactivity determined. The data shown were corrected for background radioactivity and are the means of triplicate determinations.

Figure 2. Increasing amounts of unconjugated 2B8 were analyzed for binding to human B-cells (SB) using FACS analysis. Comparisons were made with a commercially available anti-CD20 monoclonal antibody (B1) and with two irrelevant isotype antibodies. Goat anti-mouse IgG-FITC $F(ab)'_2$ was used as the secondary reagent. The results show that 2B8 is specific for the CD20 antigen and that it exhibits greater binding than B1.

Figure 3. Human B-cells (SB) were incubated with increasing amounts of ^{125}I-labeled 2B8. Triplicate samples were incubated for one hour and cell-bound radioactivity was determined after filtration to collect cells. Scatchard analysis allowed calculation of an apparent affinity constant of 4.3 X 10^{-9} M.

Figure 4. Immunoreactivity of native 2B8, 2B8-MX-DTPA, and B1. The B1 antibody was radiolabeled as described in the Methods section. Ten nanograms of radiolabeled B1 were mixed with increasing concentrations of the competitor and the mixture added to wells of V&P filter plates containing 100,000 antigen-positive SB cells each; all determinations were performed in triplicate. Following a one hour incubation at ambient temperature, the wells were washed extensively. Subsequently, the filters were dried and the associated radioactivity determined by gamma counting; all values were corrected for background. Values shown are the means of triplicate determinations.

Figure 5. Antibody 2B8 was formulated at a final concentration of 10mg/mL in normal saline or normal saline containing 10 mM glycine-HCl, pH 6.8. Duplicate sets of samples were then placed in screw-capped vials, the vials purged with nitrogen, and then capped. The samples were then incubated at 4°C or 30°C for 12 weeks; the immunoreactivity of the samples was evaluated weekly. No loss of immunoreactivity was observed with any of the 2B8 samples throughout

the 12-week study. Immunoreactivities at week 1 (Fig. 5A), week 6 (Fig. 5B) and week 12 (Fig. 5C) are depicted.

Figure 6. Binding assay for determination of immunoreactivity of ^{111}In-labeled 2B8-MX-DTPA incubated in PBS, pH 7.4 containing 50 mg/mL human serum albumin (48 h incubation). Figure 6A) A constant amount of radiolabeled antibody (5 ng/mL) was incubated with increasing volumes of SB cells (20 x 10^6 cells/mL). The amount of radioactivity (cpm) bound to cells was plotted against the volume of cell suspension added. Figure 6B) Total applied radioactivity over bound radioactivity (AT/B) was plotted. Linear extrapolation allowed calculation of the y-intercept (0.997). The reciprocal of the y-intercept X 100 yielded an immunoreactivity value of 100% at infinite antigen excess.

Figure 7. Autoradiograms obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 75 mg/mL human serum albumin and 1mM DTPA. At the indicated times, samples were electrophoresed on 4-20% Tris-glycine gels using non-reducing conditions, denaturing conditions (SDS). The samples were loaded at 5 μL (lanes 1,2), 10 μL (lanes 5,6). The gels were exposed to x-ray film for approximately 15 min at ambient temperature and photographed.

Figure 8. Densitometric scan of time zero autoradiogram obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 75 mg/mL human serum albumin and 1 mM DTPA. The sample was electrophoresed on a 4-20% Trib-glycine gel using non-reducing conditions. Samples were loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. The gel was exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#2) was 96.2%.

Figure 9. Densitometric scan of 48 h autoradiogram obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 75 mg/mL human serum albumin and 1 mM DTPA. The sample was electrophoresed on a 4-20% Tris-glycine gel using non-reducing conditions. Samples were loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. The gel was exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#2) was 95.5%.

Figure 10. Autoradiograms obtained from SDS-PAGE analysis of ^{111}In-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 50 mg/mL human serum albumin. At the indicated times, samples were electrophoresed on 4-20% Tris-glycine gels using non-reducing conditions. The samples were loaded at 5 μL (lanes 1, 2), 10 μL (lanes 3, 4), and 20 μL (lanes 5, 6). The gels were exposed to x-ray film for approximately 15 min at ambient temperature and photographed. (Note: The 48 h autoradiogram was obtained using intensifying screens resulting in a more intense signal compared to the time zero autoradiogram).

Figure 11. Densitometry scan of time zero autoradiogram obtained from SDS-PAGE analysis of ^{111}In-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 50 mg/mL human serum albumin. The sample was electrophoresed on a 4-20% Tris-glycine gel under non-reducing conditions. The sample was loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. The gel was exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#3) was 95.9%.

Figure 12. Densitometry scan of 48 h autoradiogram obtained from SDS-PAGE analysis of ^{111}In-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 50 mg/mL human serum albumin. The sample was electrophoresed on a 4-20% Tris-glycine gel under non-reducing conditions. The sample was loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. The gel was exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes scanned using a densitometer. The relative area of the radiolabeled conjugate was 97.0% (combined areas of peaks #2, 3, and 4).

Figure 13. Autoradiograms obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 37°C in human serum. At the indicated times, samples were electrophoresed on 4-20% Tris-glycine gels using non-reducing conditions. The samples were loaded at 5 μL (lanes 1, 2), 10 μL (lanes 3, 4), and 20 μL (lanes 5, 6). The gels were exposed to x-ray film for approximately 15 min at ambient temperature and photographed.

Figure 14. Densitometric scan of time zero autoradiogram obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 37°C in human serum. The sample was electrophoresed on a 4-20% Tris-glycine gel using non-reducing conditions. The sample was loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. Gels were exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#2) was 97.9%.

Figure 15. Densitometric scan of 98 h autoradiogram obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 37°C in human serum. The sample was electrophoresed on a 4-20% Tris-glycine gel using non-reducing conditions. The sample was loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. Gels were exposed to x-ray film for approximately 15 min at ambient temperature

and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#2) was 94.7%.

Figure 16. Autoradiograms obtained from SDS-PAGE analysis of ^{111}In-labeled 2B8-MX-DTPA incubate at 37°C in human serum. At the indicated times, samples were electrophoresed on 4-20% Tris-glycine gels using non-reducing conditions. The samples were loaded at 5 μL (lanes 1, 2), 10 μL (lanes 3, 4), and 20 μL (lanes 5, 6). The gels were exposed to x-ray film for approximately 16-20 h at ambient temperature and photographed.

Figure 17. Densitometric scan of time zero autoradiogram obtained from SDS-PAGE analysis of ^{111}In-labeled 2B8-MX-DTPA incubated at 37°C in human serum. The sample was electrophoresed on a 4-20% Tris-glycine gel using non-reducing conditions. The sample was loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. The gel was exposed to x-ray film for approximately 16-20 h at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#3) was 95.3%.

Figure 18. Densitometric scan of the 96 h autoradiogram obtained from SDS-PAGE analysis of ^{111}In-labeled 2B8-MX-DTPA incubated at 37°C in human serum. The sample was electrophoresed on a 4-20% Tris-glycine gel using non-reducing conditions. The sample was loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. The gel was exposed to x-ray film for approximately 16-20 h at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#3) was 94.0%.

Figure 19. Cynomolgus monkeys were injected intravenously every 48 hours for a total of seven injections; the amounts administered are shown. Circulating T- and B-cell levels were determined by FACS analysis using anti-CD2

(T-cell), anti-Mo-IgG (2B8), anti-CD20 (Leu 16), and anti-human-IgG (B-cell). No effect was observed on circulating T-cell levels. (Group V animals were given a single dose).

Figure 20. The recovery of circulating B-cell levels in animals receiving 2B8 was followed by FACS analysis using the fluorescently-labeled antibodies described in the brief description of Fig. 19. The animals in Groups III and IV were not monitored as they were sacrificed on day 13.

Figure 21. Cynomolgus monkeys were injected intravenously with ^{89}Y-2B8-MX-DTPA which had been prepared using clinical-grade 2B8-MX-DTPA. The animals were dosed every 48 hours with the amounts shown above for a total of seven doses. On days 0, 2, 7, 10 and 14 the monkeys were bled and evaluated for serum chemistries hematology and circulating B-cell levels (day 10 sera were not analyzed for B-cell content). Other than decreased total lymphocyte count in all animals, except one individual in groups II, no significant abnormalities were noted during the course of the study.

Figure 22. The clearance of murine anti-CD20 antibody 2B8 from cynomolgus monkeys was determined by ELISA following a single injection of 10 mg/kg on day zero. As shown in panel A, the antibody exhibited at β $t_{1/2}$ value of approximately 4.5 days. The clearance of the 2B8 antibody and its MX-DTPA conjugate from the circulation of BALB/c mice are shown in panel B. Mice were injected intravenously with 25 μg of native or conjugated 2B8 and blood samples taken at various times up to 264 hours following injection; sera was subsequently analyzed by enzyme immunoassay using SB cells as the capture agent. Both the native and conjugated antibodies exhibited clearance values of 8.75 days.

Figure 23. Twenty BALB/c mice were each injected with 1.1 μCi of radiolabeled conjugate (100 μL) formulated in PBS, pH 7.4, containing 50 mg/mL HSA. Groups of five mice each were sacrificed at 1, 24, 48, and 72 hours and then blood and various tissues prepared and analyzed for associated radioactivity.

Figure 24. Twenty BALB/c mice were each injected intravenously with approximately 1.0 μCi (in 100 μl) of radiolabeled conjugate formulated in 1 X PBS, pH 7.4, containing 75 mg/mL human serum albumin and 1 m MDPA. Groups of five mice each were sacrificed at 1, 24, 48 and 72 hours and their blood and various tissues prepared and analyzed for associated radioactivity.

Figure 25. Athymic mice bearing Ramos B-cell tumors were injected intravenously with 24 μCi of ^{111}-In-2B8-MX-DTPA and groups of three mice each were sacrificed at 0, 24, 48 and 72 hours. Following tissue preparation and determination of associated radioactivity, the percent injected dose per gram tissue values were determined and plotted as shown.

Figure 26. Binding assay for determination of immunoreactivity of "mix-&-shoot" ^{90}Y-labeled 2B8-MX-DTPA incubated in PBS, pH 7.4 containing 50-75 mg/mL human serum albumin (48 h incubation). Panel A) A constant amount of ^{90}Y-labeled antibody (approximately 1 ng/ml) was incubated with increasing amounts of SB cells. The amount of radioactivity (cpm) bound to cells was plotted against the cell concentration. Panel B) Total applied ^{90}Y radioactivity over bound radioactivity (AT/B) was plotted. Linear extrapolation allowed calculation of the y-intercept (1.139). The reciprocal of the y-intercept X 100 yielded an immunoreactivity value of 87.9% at infinite antigen excess. No binding was observed with CD20-negative cells (HSB).

Figure 27. Autoradiograms obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 75 mg/mL human serum albumin and 1mM DTPA. At the indicated times, samples were electrophoresed on 4-20% Tris-glycine gels using non-reducing conditions, denaturing conditions (SDS). The samples were loaded at 5 μL (lanes 1,2), 10 μL (lanes 5,6). The gels were exposed to x-ray film for approximately 15 min at ambient temperature and photographed.

Figure 28. Densitometric scan of time zero autoradiogram obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 75 mg/mL human serum albumin and 1 mM DTPA. The sample was electrophoresed on a 4-20% Trib-glycine gel using non-reducing conditions. Samples were loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. The gel was exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#2) was 96.1%.

Figure 29. Densitometric scan of 48 h autoradiogram obtained from SDS-PAGE analysis of ^{90}Y-labeled 2B8-MX-DTPA incubated at 4°C in PBS, pH 7.4 containing 75 mg/mL human serum albumin and 1 mM DTPA. The sample was electrophoresed on a 4-20% Tris-glycine gel using non-reducing conditions. Samples were loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. The gel was exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#2) was 94.1%.

Figure 30. Autoradiograms obtained from SDS-PAGE analysis of "mix-&-shoot" ^{90}Y-labeled 2B8-MX-DTPA incubated at 37°C in human serum. At the indicated times, samples were electrophoresed on 4-20% Tris-glycine gels using

non-reducing conditions. The samples were loaded at 5 μL (lanes 1, 2), 10 μL (lanes 3, 4), and 20 μL (lanes 5, 6). The gels were exposed to x-ray film for approximately 15 min at ambient temperature and photographed.

Figure 31. Densitometric scan of time zero autoradiogram obtained from SDS-PAGE analysis of "mix-&-shoot" ^{90}Y-labeled 2B8-MX-DTPA incubated at 37°C in human serum. The sample was electrophoresed on a 4-20% Tris-glycine gel using non-reducing conditions. The sample was loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. Gels were exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#2) was 89.1%.

Figure 32. Densitometric scan of 72 h autoradiogram obtained from SDS-PAGE analysis of "mix-&-shoot" ^{90}Y-labeled 2B8-MX-DTPA incubated at 37°C in human serum. The sample was electrophoresed on a 4-20% Tris-glycine gel using non-reducing conditions. The sample was loaded at 5 μL, 10 μL, and 20 μL in duplicate wells. Gels were exposed to x-ray film for approximately 15 min at ambient temperature and one of the lanes was scanned using a densitometer. The relative area of the radiolabeled conjugate peak (#2) was 88.8%.

Figure 33. Twenty BALB/c mice were each injected intravenously with 5 μCi ^{90}Y-labeled 2B8-MX-DTPA formulated in 1 X PBS, pH 7.4, containing 75 mg/mL human serum albumin and 1 mM DTPA. Groups of five mice each were sacrificed at 1, 24, 48 and 72 hours and their blood and various tissues prepared and analyzed for associated radioactivity.

Figure 34. Increasing amounts of CHO-derived 2B8 antibody labeled were incubated with a fixed concentration of freshly harvested CD20-positive B-cells (SB) or CD20-negative T-cells (HSB). Antibody binding to cells was quantified using FACS analysis using goat anti-mouse IgG-FITC F(ab)'$_2$ as described herein.

Comparison was made to an irrelevant isotype antibody (S004). Only the CHO-derived.

2B8 antibody showed any appreciable binding to CD20-positive SB cells.

Figure 35. The immunoreactivity of CHO-derived 2B8 was compared to the 2B8-49 parent antibody produced in a hybridoma cell line by direct competition in an ORIGEN assay. Increasing amounts of antibody was incubated with a fixed concentration of CD20-positive B-cells (SB) and a trace amount of ruthenium-labeled CHO 2B8. After incubation for three hours at ambient temperature, binding, expressed as relative electrochemiluminescence (ECL), was determined using the ORIGEN instrument as described in the Materials and Methods. Values represent the means of duplicate determinations. Average affinity constants for CHO 2B8 and 2B8-49 were calculated to be 1.3 X 10^{-10} M and 2.5 X 10^{-10} M, respectively. An irrelevant isotype antibody (S004), was included for comparison.

Figure 36. The binding of 2B8-MX-DTPA conjugates prepared from CHO-derived 2B8 was compared to the unconjugated antibody by direct competition in an ORIGEN assay. Conjugates were prepared by incubation of 2B8 with MX-DTPA for 8, 17, and 24 h before removal of unreacted chelate. For binding assessment, antibodies were incubated with a fixed concentration of CD20-positive B-cells (SB) and a trace amount of ruthenium-labeled CHO 2B8. After incubation for three hours at ambient temperature, binding, expressed as relative electrochemiluminescence (ECL), was determined using the ORIGEN instrument as described in the Materials and Methods. Values represent the means of duplicate determinations. Conjugate preparations exhibited similar binding compared to unconjugated 2B8 antibody.

Figure 37. A) SB cells were washed and resuspended to 90 X 10^6 cells/mL with dilution buffer (1X PBS, pH 7.4 containing 1% (w/v) bovine serum albumin.

Increasing concentrations of cells were incubated for 3 h with 7.5 ng/mL In2B8 prepared using 2B8-MX-DTPA lot 0165A. B) Double-inverse plot of cell concentration vs. bound radioactivity/total radioactivity (B/AT). Immunoreactivity was calculated as 1/y-intercept x 100. Immunoreactivity and correlation coefficient (R) values were 80.6% and 0.981, respectively.

Figure 38. A) SB cells were washed and resuspended to 90 X 10^6 cells/mL with dilution buffer (1X PBS, pH 7.4 containing 1% (w/v) bovine serum albumin. Increasing concentrations of cells were incubated for 3 h with 2 ng/mL Y2B8 prepared using 2B8-MX-DTPA lot # 0165A. B) Double-inverse plot of cell concentration vs. bound radioactivity/total radioactivity (B/AT). Immunoreactivity was calculated as 1/y-intercept x 100. Immunoreactivity and correlation coefficient (R) values were 72.2% and 0.999, respectively.

5. Detailed Description of the Invention

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

low metal - refers to reagents treated to reduce metal contamination to a level which does not impact radioincorporation

antigen positive - means expresses antigen that is recognized by particular antibody of the invention in such a way that the antibody is capable of binding.

% radioincorporation - refers to the amount of radiolabel from a radiolabeling reaction that is conjugated to the antibody relative to the total amount of radiolabel initially added to the reaction.

% binding - refers to the amount of antibody from a sample which binds to the target antigen, with or without specificity.

% immunoreactivity or binding specificity- refers to that amount of an antibody sample which binds to the target antigen with specificity.

diagnostic antibody - refers to an antibody conjugated to a radiolabel such us ^{111}I which can effect diagnostic imaging of tumors and antigen positive cells.

therapeutic antibody - refers to an antibody conjugated to a alpha or beta emitting radiolabel (such as ^{90}Y) which can effect cell killing when bound to the targeted antigen.

DESCRIPTION OF THE INVENTION

Pre-Clinical Development of Murine Monoclonal Anti-CD20 Antibody 2B8, Conjugated 2B8, ^{111}In and ^{90}Y-Labeled 2B8

I. Materials and Methods for Development of Murine Monoclonal Anti-CD20 Antibody 2B8, Conjugate 2B8-MX-DTPA, ^{111}In-Labeled 2B8-MX-DTPA and HPLC-Purified ^{90}Y-MX-DTPA

A. Materials.

1. Cells.

The human cell lines SB and HSB were obtained from the American Type Culture Collection and cultured in RPMI-1640 containing 10% fetal bovine serum. The CD20-positive SB cell line is a B lymphoblastoid cell line derived from the peripheral blood buffy coat of a patient with acute lymphoblastic leukemia (1). The antigen-negative cell line HSB is a T lymphoblastoid cell line developed from tumors induced in newborn Syrian hamsters (2). The murine myeloma cell line SP2/0 was similarly maintained in RPMI-1640 containing 10% fetal bovine serum.

2. Antibodies.

The anti-CD20 antibodies Bl and Leu 16 were purchased from Coulter Immunology and Becton/Dickinson, respectively. The ^{125}I-labeled goat anti-mouse IgG and goat anti-human IgG antibodies were obtained from ICN. Goat F(ab')2 anti-mouse IgG was obtained from Cappel.

3. Reagents.

Freund's complete and incomplete adjuvants were purchased from Sigma Chemical Company. Polyethylene glycol, HAT concentrate, and HT concentrate were all obtained from Boehringer Mannheim. Fluorescein isothiocyanate (FITC) was purchased from Sigma Chemical Company. Indium-[111] chloride and ^{90}Y chloride were obtained from Amersham or NEN Dupont. Yttrium-[89] chloride was purchased from Aldrich Chemical Company. All other reagents were obtained from standard sources.

Reagents used for conjugation and radiolabeling protocols were processed to remove contaminating heavy metal ions which could compete with the radioisotopes during the radiolabeling step. Reagents were typically processed by passing the solutions through a column of Chelex 100 ion exchange resin (BioRad Industries) or batch processing by addition of Chelex 100 to a prepared solution. Low metal-containing water, either Milli-Q-purified or Water for Irrigation (WFIr) was used for all preparations and dilutions. The metal-free solutions were sterile-filtered and collected in sterile plastic containers.

B. Methods.

1. Production and Screening of 2B8 Hybridoma Supernatants by RIA.

Ten BALB/c mice were immunized with 20 million SB cells suspended in PBS containing Freunds complete adjuvant. The cells were injected both s.c and i.p at multiple sites on the animal. After a 2 week rest period the mice were injected a second time with SB cells emulsified in Freund's incomplete adjuvant.

Subsequent immunization boosters were performed on a weekly schedule with SB cells suspended in PBS. Mice were immunized for a period of 6 weeks to 4 months.

Two animals at a time were sacrificed by cervical dislocation and their spleens removed for fusion with the murine myeloma SP2/0. Animals were chosen based on the ability of post-immune sera to effectively inhibit the binding of radiolabeled Coulter Bl anti-CD20 antibody to human SB cells. Three days prior to each fusion the selected animals were given one last intravenous (tail vein) injection of 20 million SB cells in PBS. Upon sacrifice the spleens were removed under aseptic conditions and the splenocytes fused with SP2/0 cells at a ratio of 5:1 (splenocytes:SP2/0). Fused cells were washed in tissue culture media and distributed into 96 well plates containing HAT selection media. Hybridomas were screened by inhibition radioimmunoassay using Coulter B1 antibody after 10-14 days.

Screening of hybrids secreting anti-CD20 antibody was accomplished using established radioimmunoassay methods. Briefly, Coulter Bl anti-CD20 antibody was purified by Protein A affinity chromatography. Fifty micrograms of purified antibody was coupled to ^{125}I by brief oxidation in the presence of Iodobeads (Pierce Chemical Co.), following the manufacturer's procedure. The radiolabeled antibody was desalted on amberlite resin and stored in dilution buffer (PBS, pH 7.4, containing 0.2% gelatin, 0.02% sodium azide, and 1.0% BSA). Ten nanograms of radiolabeled antibody was placed in each well of a previously blocked filter assay plate (blocking buffer: dilution buffer containing 10% FBS) along with 50 μL of hybridoma supernatant from test wells and 100,000 SB cells suspended in 50 μL dilution buffer. The suspension was incubated for one hour at ambient temperature. The plates were washed thoroughly with wash buffer (PBS, pH 7.4, containing 0.2% gelatin and 0.02% sodium azide) on a V&P Scientific vacuum manifold and filter bottoms containing trapped SB cells were transferred to a gamma counter. Wells containing only HAT media and labeled Bl antibody were

used as background controls and identical wells containing SB cells were used as positive controls. Inhibition controls contained radiolabeled Bl and various amounts of unlabeled Bl antibody ranging from 2 μg to 2 ng.

2. Flow Cytometry Studies.

a. Cell Lines

Preliminary flow cytometry studies were performed with supernatants from 2B8 hybridoma cultures. One hundred microliters of hybridoma supernatant was incubated with SB cells for one hour at ambient temperature; a secondary antibody (goat F(ab')2 anti-mouse IgG; Cappel), used at a 1/400 dilution, was added subsequently and the incubation continued for 1 hour in the dark. The cells were washed for 5 times. Controls included cells only (no primary or secondary antibody) from which autofluorescence was determined, cells with secondary antibody only to determine non-specific binding and commercially available fluorescein isothiocyanate-conjugated Bl (Bl-FITC) for a CD20 population control.

For some experiments, fluorescein was coupled to purified 2B8 antibody through the reaction of amino groups with fluorescein isothiocyanate (FITC). Briefly, 2B8 antibody (1.2 mg/mL) was incubated in pH 9.5, 0.1M sodium carbonate buffer with 150-200 μg FITC per mg protein. The solution was incubated at room temperature for 2 hours and the resulting 2B8-FITC conjugate was purified on a Sephadex G-25 column. Other reagents used in these studies such as Bl and Leu 16 were purchased as fluorescein conjugates directly from Coulter or Becton Dickinson.

Cells to be analyzed were harvested and washed three times with PBS containing 0.2% BSA and 0.1% sodium azide. Viability was determined by trypan blue exclusion with a viability requirement of >90%. Cell concentrations were adjusted to 3 million per ml with 50 μL added per well into 96 well U-bottom plates. Primary antibody (50 μL) was added to appropriate wells and the mixture incubated for 30 min. to 1 h. at ambient temperature; subsequently the cells were

washed 5 times with 200 μL/well of PBS containing 0.2% BSA and 0.02% sodium azide. Cells were centrifuged in the plates at 1300 RPM for 1 min. in a Sorvall centrifuge and the supernatants removed by gently "flicking" the plates. Secondary antibody, if needed, was added and incubated for an additional 30 min to 1h at ambient temperature in the dark; wells were then washed as above. Finally, 200 μL of fixing buffer (0.15 M sodium chloride containing 1% paraformaldehyde, pH 7.4) was added to each sample and the treated cells transferred to 12X75 mm tubes for analysis.

b. <u>Whole Blood From Cynomolgus Monkeys.</u>

After removal of plasma, the cells were washed twice by centrifugation and resuspension in HBSS. Fetal bovine serum (2 mL) was added and the cells resuspended. One hundred microliters of the resuspended cells were then distributed to each of 6, 15 ml conical centrifuge tubes. Fluorescently-labeled monoclonal antibodies were added as follows:

Tube #1:	Murine anti-CD2-FITC (AMAC), 2.5 μg/mL, 5 μg;
Tube #2:	Goat anti-Human IGM-FITC (Fisher) 2.5 μg/mL, 5 μg;
Tube#3:	Goat anti-mouse IgG-RPE (Fisher) 2.5 μg/mL, 5 μg;
Tube #4:	Goat anti-Human IgM-FITC + Goat anti-mouse IgG-RPE (absorbed), 2.5 μg/mL, 5 μg;
Tube #5:	anti-human CD20-FITC (anti-Leu 16, Becton Dickinson), 5μg;
Tube #6:	Cells only (auto-fluorescence).

Labeled antibodies and cells were centrifuged for 2 min at 1500 rpm to mix cells and antibodies and all 6 samples were then placed on ice and incubated for 30 min. Subsequently the tubes were removed from the ice and lysing buffer (prewarmed to 37°C) was added to a volume of 12 mL. The samples were then incubated for 15 min at room temperature, centrifuged for 5 min at 4°C at 1500 rpm, and the supernatants removed. Cell pellets were then washed twice in labeling buffer (PBS containing 1% BSA and 0.05% sodium azide).

Subsequently the cells were fixed by the addition of 0.5 mL of fixation buffer (0.15 M sodium chloride, pH 7.4, containing 1% paraformaldehyde) per tube and analyzed on a Becton Dickinson FACScan instrument using autocompensation and precalibration with Calibrite beads. Green fluorescence from fluorescein was measured in FL1 mode and red fluorescence from phycoeretherin was measured in FL2 mode. Data were expressed in log form. Viable lymphocyte populations were initially identified by forward vs. right angle light scatter in a dot plot bitmap. The total lymphocyte population was then isolated by gating out all other events. Subsequent fluorescence measurements reflected only those specific events which occurred within the gated area.

For high-dose pharmacology/toxicology studies the pre-study lymphocyte levels were determined for each cynomolgus monkey and used as baseline values. The percentage of T- and B-cells and T:B ratios were calculated and used as depletion references. The pre-study B cell population was enumerated with Leu 16 and anti-human IgM antibodies.

After injection of 2B8 into the monkeys, when the CD20 antigen was saturated with 2B8, the percentage of B cells in the total population was approximated using goat anti-human IgM-FITC, anti-mouse IgG-RPE and the double staining population containing these two markers. The double staining population was used for quantitation until all of the 2B8 was cleared from the peripheral blood of the animals. The percentage of T cells in the total lymphocyte population was estimated using anti-CD2-FITC. Data were averaged from three,

10,000 event measurements made with each sample. Cells from each of the designated blood samples were evaluated subsequently, enumerating in each case the T- and B-cell subpopulations within the total lymphocyte population. The T:B ratios were also examined. Depletion of B-cells was calculated as the percent of reduction of B-cells relative to original B-cell levels for each individual monkey.

3. Radioiodination and Immunoprecipitation of CD20.

One hundred million SB cells were divided into two equal parts after surface iodination with ^{125}I and Iodobeads (Pierce Chemical Co.). The cells were washed repeatedly by centrifugation until radioactivity levels in the supernatant returned to background. One hundred micrograms of 2B8 or Bl (Coulter Immunology) antibody were added to either of the two samples of labeled B cells. The antibodies and SB cells were incubated overnight and then washed three times by centrifugation until all of the unbound antibody was removed. The cell pellets containing bound 2B8 and Bl were then lysed and extracted by addition of 1% NP-40 detergent in 0.1 M Tris-HC1, pH 8.0, followed by incubation at room temperature for 1 h. The extract was centrifuged in a microfuge at high speed for 30 min and the supernatants were transferred to new tubes. Protein A-Sepharose (300 μL) was added to each tube and the resin pelleted by centrifugation. The protein A-Sepharose was then washed 20 times to remove non specifically bound iodinated protein. When the bead-to-supernatant radioactivity ratio reached a value of 100, the pellet was extracted with SDS PAGE sample buffer and heated to boiling. After cooling, approximately 15,000 cpm of each of the extracts were added to wells of a 10% polyacrylamide gel. A low molecular weight pre-stained standard (BioRad Inc.) was added to a separate well and used for molecular weight estimation. The proteins were resolved by electrophoresis and the gel was dried and exposed to a sheet of X-ray film for 24 hours at -70°C; subsequently the film was developed and analyzed.

4. Scatchard Analysis of 2B8 Binding.

Purified 2B8 was evaluated for apparent affinity by Scatchard analysis. Radiolabeled 2B8 was prepared by reaction with ^{125}I in the presence of Iodobeads. Following removal of free iodine the radiolabeled antibody was incubated in various concentrations, in duplicate, ranging from 5000 ng per well to 35 ng/well with 10,000 SB cells. The amount of antibody binding to cells was calculated from the specific activity of the ^{125}I-labeled 2B8. The ratio of bound/free antibody was plotted against the molar concentration of bound antibody and the apparent affinity constant was determined from the ratio of the X and Y axis intercepts.

5. Preparation of 2B8-MX-DTPA

a. Source of MX-DTPA

For some pre-clinical studies, carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylenetriaminepentaacetic acid (MX-DTPA) was provided as a dry solid by Dr. Otto Gansow at the National Institute of Health and stored desiccated at 4°C protected from light. Stock solutions of the chelate were prepared in Milli-Q water and the concentration determined by assessing the radioactivity and using the specific activity of the compound. Stock solutions were generally 2-5 mM and were stored at -70°C in polypropylene tubes. For other studies, MX-DTPA was obtained from Coulter Immunology as the disodium salt in water and stored at -70°C.

b. Maintenance of Metal-Free Conditions

In addition to using metal-free reagents, all manipulations of reagents were performed so as to minimize the possibility of metal contamination. When possible, polypropylene plastic containers such as flasks, beakers and graduated cylinders were used. These were washed with Alconox and exhaustively rinsed with Milli-Q water before use. In addition, metal-free pipette tips (BioRad) were used for accurately manipulating small volumes. For manipulating larger volumes of reagents, sterile, plastic serological pipettes (available in 1 to 25 mL sizes) were

used. Reactions were conveniently performed in screw-top, polypropylene microfuge tubes (Sardstedt Industries; 1.5 mL capacity) or polypropylene conical tubes (Costar; 15 mL and 50 μL). When dialysis tubing was manipulated, disposable surgical gloves, previously rinsed with Milli-Q water, were worn.

c. Preparation of Antibody

The murine anti-CD20 antibody 2B8 was purified initially from ascites by Protein A and QAE chromatography. For later experiments 2B8 was purified from hollow-fiber bioreactor supernatants using the same purification process. The hollow-fiber-derived antibody has now been replaced for commercialization purposes with the CHO-derived antibody described in Example 2.

The antibody was prepared for conjugation by transferring it into metal-free 50 mM bicine-NaOH, pH 8.6, containing 150 mM NaCl, using dialysis or repetitive buffer exchange. In some studies, buffer exchange was effected using repetitive ultrafiltration with Centricon 30 (Amicon) spin filters (30,000D MWCO). In general, 50-200 μL of protein (10 mg/mL) was added to the filter unit and 2 mL of bicine buffer added. The filter was centrifuged at 4°C in a Sorval SS-34 rotor at 6,000 rpm for 45 min. Retentate volume was approximately 50-100 μL. This process was repeated twice with the same filter. Retentate was transferred to a polypropylene 1.5 mL screw cap tube, assayed for protein, diluted to 10.0 mg/mL and stored at 4°C until used for conjugation. For some studies, the protein was transferred into 50 mM sodium citrate, pH 5.5 containing 150mM NaCl and 0.05% sodium azide using the same protocol described above.

d. Conjugation Protocol

Conjugation of 2B8 with MX-DTPA was performed in polypropylene tubes at ambient temperature. Frozen stock solutions of MX-DTPA were thawed immediately before use. Typically, 50-200 μL of antibody at 10 mg/mL were reacted with chelate at a molar ratio of chelate-to-protein of 4:1. Reactions were

initiated by adding the chelate stock solution and gently mixing; the conjugation was allowed to proceed overnight, generally for 14 to 20 h, at ambient temperature. Unreacted chelate was removed from the conjugate by dialysis or repetitive ultrafiltration, as described above, into metal-free normal saline (0.9% w/v) containing 0.05% sodium azide. The protein concentration was adjusted to 10 mg/mL and stored at 4°C in a polypropylene tube until radiolabeled.

e. Determination of Chelate Incorporation

Chelate incorporation was determined by scintillation counting and comparing the value obtained with the purified conjugate to the specific activity of the carbon-[14]-labeled chelate. For later studies, in which non-radioactive chelate obtained from Coulter Immunology was used, chelate incorporation was assessed by incubating the conjugate with an excess of a radioactive carrier solution of ^{90}Y of known concentration and specific activity.

Briefly, a stock solution of yttrium chloride of known concentration was prepared in metal-free 0.05 N HCl to which carrier-free ^{90}Y (chloride salt) was added. An aliquot of this solution was analyzed by liquid scintillation counting to determine an accurate specific activity for this reagent. A volume of the yttrium chloride reagent equal to 3-times the number of mols of chelate expected to be attached to the antibody, typically 2 mol/mol antibody, was added to a polypropylene tube, and the pH adjusted to 4.0-4.5 with 2 M sodium acetate. Conjugated antibody was subsequently added and the mixture incubated 15-30 min at ambient temperature. The reaction was quenched by adding 20 mM EDTA to a final concentration of 1 mM and the pH of the solution adjusted to approximately pH 6 with 2M sodium acetate.

After a 5 min incubation the entire volume was purified by high-performance size-exclusion chromatography as described below. The eluted protein-containing fractions were combined, the protein concentration determined, and an aliquot assayed for radioactivity. The chelate incorporation was calculated

using the specific activity of the ^{90}Y chloride preparation and the protein concentration.

f. Immunoreactivity of 2B8-MX-DTPA

The immunoreactivity of conjugated 2B8 was assessed using whole-cell ELISA. Mid-log phase SB cells were harvested from culture by centrifugation and washed two times with 1X HBSS. Cells were diluted to 1-2 X 10^6 cells/mL in HBSS and aliquoted into 96-well polystyrene microliter plates at 50,000-100,000 cells/well. The plates were dried under vacuum for 2 h at 40-45°C to fix the cells to the plastic. The plates were stored dry at -20°C until used. For assay, the plates were warmed to ambient temperature immediately before use, then blocked with 1X PBS, pH 7.2-7.4 containing 1% BSA (2 h). Samples for assay were diluted in 1X PBS/1% BSA, applied to plates and serially diluted (1:2) into the same buffer. After incubating plates for 1 h at ambient temperature, the plates were washed three times with 1X PBS. Secondary antibody (goat anti-mouse IgGl-specific HRP conjugate) (50 μL) was added to wells (1:1500 dilution in 1X PBS/1% BSA) and incubated 1 h at ambient temperature. Plates were washed four times with 1X PBS followed by the addition of ABTS substrate solution (50 mM sodium citrate, pH 4.5 containing 0.01% ATBS and 0.001% H_20_2). Plates were read at 405 nm after 15-30 min incubation. Antigen-negative HSB cells were included in assays to monitor non-specific binding. Immunoreactivity of the conjugate was calculated by plotting the absorbance values vs. the respective dilution factor and comparing these to values obtained using native antibody (representing 100% immunoreactivity) tested on the same plate. Several values on the linear portion of the titration profile were compared and a mean value determined.

g. *In Vitro* Stability of Native 2B8 and 2B8-MX-DTPA

For this 12-week assessment of antibody and conjugate stability, aliquots of 2B8 antibody and 2B8-MX-DTPA were formulated in either normal saline or normal saline containing 10 mM glycine-HC1, pH 6.8. Duplicate sets of samples were incubated at both 4° and 30°C and samples assayed weekly using the following methods: SDS-PAGE (both reducing and nonreducing), immunoreactivity by whole-cell enzyme immunoassay using either SB (antigen-positive) or HSB (antigen-negative) cells as capture, and isoelectric focusing gel electrophoresis (pH range, 3-10). In addition, the radiolabeling efficiency of the conjugate was assessed at weeks 4, 8, and 12 by radiolabeling the conjugate with ^{90}Y and analyzing the product by SDS-PAGE and autoradiographic analysis. Finally, in a separate study, aliquots of 2B8-MX-DTPA incubated at 4° and 30°C for 10 weeks were radiolabeled with ^{111}In and evaluated in a biodistribution study in BALB/c mice as described below.

h. Immunohistology Studies.

Immunohistology studies with both the native and conjugated (2B8-MX-DTPA) antibodies were performed by IMPATH Laboratories using sections of human tissues fixed with acetone. The antibody was purified from hollow-fiber bioreactor supernatants by chromatography on protein A and Q Sepharose. Clinical-grade conjugate was prepared using MX-DTPA from Coulter Immunology according to the protocol described above.

i. *In Vitro* Immunoreactivity of Radiolabeled 2B8-MX-DTPA.

For some experiments, the whole-cell ELISA protocol used for unlabeled 2B8-MX-DTPA was used. In later experiments, immunoreactivity of ^{111}In and ^{90}Y-labeled conjugates (each prepared at IDEC Pharmaceuticals or, alternatively, at MPI Pharmacy Services, Inc.) was determined using a modified version of the whole-cell binding assay described by Lindmo (3). Briefly, increasing

concentrations of mid-log phase, antigen-positive SB cells or antigen-negative HSB cells [20-30 X 10^6 cells/mL in dilution buffer (PBS, pH 7.4 containing 1% BSA, 0.1% gelatin, and 0.02% sodium azide)] were added to duplicate sets of tubes. The radiolabeled conjugate was diluted to a final antibody concentration of 1-5 ng/mL with dilution buffer and 0.35 mL was added to each tube. Following a 75-90 min incubation period at ambient temperature the cells were pelleted by centrifugation and the supernatants collected. Radioactivity remaining in the supernatant fraction was determined with a gamma or scintillation counter. The data were plotted as the quotient of the total radioactivity added divided by the cell-associated radioactivity, versus the inverse of the cell number per tube. The y axis intercept thus represents the immunoreactive fraction.

j. <u>*In Vitro* Stability of Radiolabeled 2B8-MX-DTPA in Human Serum.</u>

The *in vitro* stability of ^{111}In- and ^{90}Y-labeled 2B8-MX-DTPA was assessed by incubation in human serum at 37°C for 96 hours. The conjugated antibody was prepared and radiolabeled with ^{111}In ("mix-and-shoot" protocol) or ^{90}Y as described above. The specific activities of the ^{111}In and ^{90}Y-labeled conjugates were 2.5 and 14.6 mCi/mg, respectively; the radiolabeled conjugates were suspended in buffer containing 75 mg/mL human serum albumin (HSA) and 1 mM DTPA (yttrium-labeled conjugate) or buffer containing 50mg/mL HSA (indium-labeled conjugate). The radiolabeled conjugates were diluted 1:10 with normal human serum (non-heat-inactivated) and aliquots placed aseptically into sterile capped tubes; these tubes were then incubated at 37°C for periods up to 96 hours. At selected times conjugate samples were removed and analyzed by non-reducing SDS-PAGE in 4-20% gradient gels followed by autoradiography, and by instant thin layer chromatography.

k. *In Vitro* Stability of Clinically-Formulated ^{111}In- 2B8-MX-DTPA.

The 2B8-MX-DTPA conjugate was radiolabeled with ^{111}In and used without HPLC purification ("mix-and-shoot" protocol). The radiolabeled antibody was diluted into PBS and human serum albumin (HSA) added to a final concentration of 50 mg/mL. The specific activity of the formulated radiolabeled conjugate was 2.2 mCi/mg. The formulated conjugate was subsequently incubated at 4°C for 48 hours and aliquots analyzed at time 0, 24 h and 48 hours using non-reducing SDS-PAGE in 4-20% gradient gels followed by autoradiography, and by instant thin layer chromatography. The immunoreactivity at each time point was assessed using the whole-cell suspension assay described in section 1 above.

l. In Vitro Stability of Clinically-Formulated ^{90}Y-2B8-MX-DTPA.

The 2B8-MX-DTPA conjugate was radiolabeled with ^{90}Y and purified by size-exclusion chromatography on HPLC using 1X PBS as an elution buffer. The radiolabeled conjugate fractions were pooled and human serum albumin and DTPA were added to final concentrations of 75 mg/mL and 1 mM, respectively. The specific activity of the formulated radiolabeled conjugate was 14.6 mCi/mg. The formulated conjugate was subsequently incubated at 4°C for 48 hours and aliquots analyzed at time 0, 24 h and 48 hours using non-reducing SDS-PAGE in 4-20% gradient gels followed by autoradiography, and instant thin layer chromatography. Immunoreactivity at each time point was assessed using the whole-cell suspension assay described in section 1 above.

2. Animal Studies.

a. Primate High Dose Pharmacology/Toxicology Study Using 2B8.

Antibody 2B8 was evaluated in a high-dose pharmacology study performed under GLP regulations at White Sands Research Center (Study Number 920111). Adult Macaca fascicularis (cynomolgus) monkeys were used; study groups each consisted of one male and one female. The antibody was injected intravenously every 48 hours for a total of seven injections. The study consisted of five groups: Group I (saline); Group II (0.6 mg/kg); Group III (2.5 mg/kg); Group IV (10 mg/kg); and, Group V (10 mg/kg on day 0 only).

Prior to initiation of the study, blood was obtained from all 10 animals and used to determine reagent backgrounds and initial B cell populations. All subsequent blood samples were drawn prior to each antibody injection. Groups III and IV were sacrificed at day 13 for complete necropsy and histopathology.

Animals in groups I, II, and V were bled on days 0, 1, 3, 7, 13, 21, 37 and 52; approximately 5 mL whole blood was drawn in heparinized tubes. Whole blood was kept at 4°C and analyzed within 24 hours. Blood from each animal was centrifuged at 2000 rpm for 5 min. and the supernatant plasma was removed for assay of serum 2B8 levels by RIA (see RIA procedure for specific assay methods). The pelleted material containing PBLs and RBCs was resuspended in FCS for FACS analysis.

b. Pharmacokinetic Studies with 2B8 and 2B8-MX-DTPA.

The mean serum beta half life of 2B8 in cynomolgus monkeys was determined using Group V animals (above). Goat anti-mouse IgGl (Fisher Scientific) was diluted to 2.0 μg per ml in 10 mM borate buffer, pH 9.6, and 50 μL was added to each well of a 96-well plate. The antibody was allowed to bind to the plate during an overnight incubation at 4°C, or for 2 h at ambient temperature. Each plate was blocked for 30 min. at ambient temperature with 150 μL per well of PBS containing 1% BSA. The plates were washed with distilled water and serum or plasma samples were applied in triplicate to individual wells at 1:100 initial dilution followed by serial 1:2 dilutions. Purified 2B8 was added to pre-

bleed sera and diluted for use as a standard curve beginning with 0.5 mg/mL; samples were diluted 1:100 and then serially diluted as with the other samples. The plates were incubated for 1 h at ambient temperature and washed 4 times with distilled water. The secondary reagent (goat anti-mouse IgGl-HRPO) was then added at 1:4000 dilution and incubated at ambient temperature for an additional hour. The plates were washed again in distilled water and 0.1 mL peroxidase substrate was added containing hydrogen peroxide. Color was allowed to develop from the reaction for 20 min.; the absorbance was subsequently determined at 405 nm using a microplate ELISA reader. The results were plotted in μg antibody per mL serum.

In addition, the β $t_{1/2}$ values of 2B8 and 2B8-MX-DTPA were determined in BALB/c mice. Unconjugated 2B8 stored at -70°C in 1X PBS, pH 7.4/10% glycerol was thawed, diluted to 0.5 mg/mL and sterile filtered. Conjugated antibody was prepared following standard protocols but with carbon-[14]-labeled chelate; chelate incorporation was 1.5 mol/mol antibody. The purified conjugate was diluted to 0.5 mg/mL in normal saline (0.9%), sterile filtered, and stored at 4°C with the native antibody until used.

Six-to-eight week old mice were injected with 100 μL of purified 2B8 antibody at a concentration of 250 μg/mL. Mice were subsequently bled by retro-orbital puncture at various times ranging from 0 to 264 hours and their sera analyzed for the presence of the native and conjugated 2B8 antibody by whole-cell enzyme immunoassay using the antigen-positive B-cell line SB as the capture. The resulting data were plotted as the concentration of 2B8 or 2B8-MX-DTPA versus time; from these results a linear regression plot was generated and the slope used to determine the β $t_{1/2}$ values.

c. <u>Pharmacology/Toxicology Study of [89]-Y-2B8-MX-DTPA in Cynomolgus Monkeys.</u>

Yttrium-[89]-bearing 2B8-MX-DTPA was prepared using the protocol described for insertion of ^{90}Y, except that HPLC purification was not used. The non-radioactive, metal-bearing conjugate was formulated in 1X PBS containing 75 mg/mL HSA and 1 mM DTPA and evaluated in GLP study number 920611 at White Sands Research Center. One male and one female monkey were included in each of four groups. The animals were injected intravenously every 48 hours for a total of 7 injections with the following amounts of drug: group 1 (saline); group II (0.003 mg/kg); group III (0.03 mg/kg); and, group IV (0.3 mg/kg). The animals were evaluated during the study by determining body weights and temperatures, food and water consumption, elimination, serum chemistries, hematology, urinalysis, and physical examinations. Animals in groups I through IV were bled prior to infusion on days 0, 2, 7, 10 and 14 and the blood analyzed for circulating B-cell levels by FACS analyses.

d. <u>Biodistribution of Radiolabeled 2B8-MX-DTPA</u>

In a preliminary study ^{111}In-labeled 2B8-MX-DTPA was evaluated for tissue biodistribution in six-to-eight week old BALB/c mice. The radiolabeled conjugate was prepared using clinical-grade 2B8-MX-DTPA following the "mix and shoot" protocol described above. The specific activity of the conjugate was 2.3 mCi/mg and the conjugate was formulated in PBS, pH 7.4 containing 50mg/mL HSA. Mice were injected intravenously with 100 μL of ^{111}In-labeled 2B8-MX-DTPA (approximately 21 μCi) and groups of three mice were sacrificed by cervical dislocation at 0, 24, 48, and 72 hours. After sacrifice, the tail, heart, lungs, liver, kidney, spleen, muscle, and femur were removed, washed, weighed; a sample of blood was also removed for analysis. Radioactivity associated with each specimen was determined by gamma counting and the percent injected dose per gram tissue subsequently determined. No attempt was made to discount the activity contribution represented by the blood associated with individual organs.

In a separate protocol, aliquots of 2B8-MX-DTPA incubated at 4° and 30°C for 10 weeks were radiolabeled with ^{111}In to a specific activity of 2.1 mCi/mg for both preparations. These conjugates were then used in biodistribution studies in mice as described above.

For dosimetry determinations, 2B8-MX-DTPA was radiolabeled with ^{111}In to a specific activity of 2.3 mCi/mg and approximately 1.1 μCi was injected into each of 20 BALB/c mice. Subsequently, groups of five mice each were sacrificed at 1, 24, 48 and 72 hours and their organs removed and prepared for analysis. In addition, portions of the skin, muscle and bone were removed and processed for analysis; the urine and feces were also collected and analyzed for the 24-72 hour time points.

Using a similar approach, 2B8-MX-DTPA was also radiolabeled with ^{90}Y and its biological distribution evaluated in BALB/c mice over a 72-hour time period. Following purification by HPLC size exclusion chromatography, four groups of five mice each were injected intravenously with approximately 1 μCi of clinically-formulated conjugate (specific activity: 12.2 mCi/mg); groups were subsequently sacrificed at 1, 24, 48 and 72 hours and their organs and tissues analyzed as described above. Radioactivity associated with each tissue specimen was determined by measuring bremstrahlung energy with a gamma scintillation counter. Activity values were subsequently expressed as percent injected dose per gram tissue or percent injected dose per organ. While organs and other tissues were rinsed repeatedly to remove superficial blood, the organs were not perfused. Thus, organ activity values were not discounted for the activity contribution represented by internally associated blood.

e. Tumor Localization of ^{111}In-Labeled 2B8-MX-DTPA.

The localization of radiolabeled 2B8-MX-DTPA was determined in athymic mice bearing Ramos B-cell tumors. Six-to-eight week old athymic mice were injected subcutaneously (left-rear flank) with 0.1 mL of RPMI-1640 containing 1.2

X 10^7 Ramos tumor cells which had been previously adapted for growth in athymic mice. Tumors arose within two weeks and ranged in weight from 0.07 to 1.1 grams. Mice were injected intravenously with 100 μL of ^{111}In-labeled 2B8-MX-DTPA (16.7 μCi) and groups of three mice were sacrificed by cervical dislocation at 0, 24, 48, and 72 hours. After sacrifice the tail, heart, lungs, liver, kidney, spleen, muscle, femur, and tumor were removed, washed, weighed; a sample of blood was also removed for analysis. Radioactivity associated with each specimen was determined by gamma counting and the percent injected dose per gram tissue determined.

3. Dosimetry Calculations

Using the biodistribution data obtained using BALB/c mice injected with either the ^{111}In or ^{90}Y-labeled 2B8-MX-DTPA (Tables 1-4 and 5-8), estimates of the radiation dose absorbed from a 1.0 mCi dose administered to a 70 Kg patient were calculated using the approach formalized by Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine. The biological half-lives of the radiolabeled conjugates were determined from the injected dose per organ values determined from the biodistribution data for each radioimmunoconjugate. For some tissues, e.g. blood, it was assumed that the biological decay of the radioconjugate followed a two-compartment model with an exponential decay from these compartments. For other tissues, e.g. the liver, whose activity levels remained roughly constant throughout the 72-hour biodistribution study, it was assumed that the biological half-life was very long and assigned a value of 1000 hours.

Table 1

Distribution of Activity 1.0 Hour Following I.V. Injection of ^{111}In-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.47 ± 0.17	40.3 ± 5.32	58.4 ± 3.1
Heart	0.087 ± 0.01	5.88 ± 0.76	0.51 ± 0.05
Lung (2)	0.149 ± 0.01	14.2 ± 1.4	2.10 ± 0.17
Kidney (1)	0.127 ± 0.02	9.82 ± 0.86	1.22 ± 0.12
Liver	1.06 ± 0.20	10.32 ±1.58	10.76 ± 1.93
Spleen	0.090 ± 0.01	6.94 ± 1.17	0.61 ± 0.03
Muscle	8.39 ± 0.98	0.70 ± 0.25	5.67 ± 1.35
Bone	3.15 ± 0.35	2.97 ± 0.71	9.10 ± 1.09
Skin	3.15 ± 0.35	0.96 ± 0.29	3.0 ± 1.12
GI Tract	2.58 ± 0.31	6.10 ± 2.00	7.80 ± 1.80
Urine			--
Feces			--
		TOTAL	99.04 ± 4.8

No. Mice = 5

Mean Weight = 20.97 ± 2.46 grams

Table 2

Distribution of Activity 24 Hours Following I.V. Injection of ^{111}In-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.47 ± 0.07	21.97 ± 1.87	32.22 ± 1.35
Heart	0.128 ± 0.03	4.02 ± 0.23	0.38 ± 0.01
Lung (2)	0.152 ± 0.02	7.90 ± 1.61	1.20 ± 0.18
Kidney (1)	0.128 ± 0.01	5.94 ± 0.40	0.76 ± 0.04
Liver	1.11 ± 0.10	10.08 ± 1.83	11.20 ± 2.23
Spleen	0.082 ± 0.01	5.04 ± 0.75	0.40 ± 0.02
Muscle	8.41 ± 0.38	1.24 ± 0.05	10.44 ± 0.76
Bone	3.15 ± 0.14	2.02 ± 0.33	6.31 ± 0.81
Skin	3.15 ± 0.14	3.75 ± 0.39	11.77 ± 1.09
GI Tract	2.91 ± 0.27	4.50 ± 0.52	6.65 ± 0.56
Urine			0.98
Feces			2.54
		TOTAL	87.10 ± 1.68

No. Mice = 5

Mean Weight = 21.03 ± 0.94 grams

Table 3

Distribution of Activity 48 Hours Following I.V. Injection of ^{111}In-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.45 ± 0.13	22.41 ± 3.95	31.90 ± 2.89
Heart	0.090 ± 0.01	4.05 ± 0.94	0.36 ± 0.06
Lung (2)	0.155 ± 0.02	8.45 ± 0.53	1.31 ± 0.19
Kidney (1)	0.125 ± 0.01	6.16 ±1.15	0.76 ± 0.07
Liver	1.040 ± 0.11	9.41 ± 2.33	9.84 ± 3.18
Spleen	0.082 ± 0.01	5.32 ± 0.71	0.48 ± 0.11
Muscle	8.26 ± 0.77	1.42 ± 0.58	11.62 ± 4.67
Bone	3.10 ± 0.29	2.08 ± 0.16	6.41 ± 0.44
Skin	3.10 ± 0.29	3.43 ± 0.59	10.54 ± 1.69
GI Tract	2.96 ± 0.20	5.05 ± 0.63	7.46 ± 0.60
Urine			1.46
Feces			6.41
		TOTAL	88.49 ± 6.87

No. Mice = 5

Mean Weight = 20.65 ± 1.93 grams

Table 4

Distribution of Activity 72 Hours Following I.V. Injection of ^{111}In-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.52 ± 0.06	18.97 ±1.31	28.51 ± 2.03
Heart	0.094 ± 0.01	3.71 ± 0.31	0.35 ± 0.04
Lung (2)	0.161 ± 0.01	7.60 ± 0.30	1.18 ± 0.09
Kidney (1)	0.135 ± 0.01	5.55 ± 0.53	0.76 ± 0.09
Liver	1.11 ± 0.11	9.90 ± 1.77	11.00 ± 2.03
Spleen	0.095 ± 0.01	5.12 ± 0.75	0.48 ± 0.04
Muscle	8.58 ± 0.34	1.04 ± 0.09	8.95 ± 0.68
Bone	3.22 ± 0.12	1.73 ± 0.34	6.04 ± 0.51
Skin	3.22 ± 0.12	3.16 ± 0.60	10.19 ± 2.03
GI Tract	2.79 ± 0.19	4.53 ± 0.83	6.37 ± 1.38
Urine			2.49
Feces			11.50
		TOTAL	87.80 ± 4.79

No. Mice = 5

Mean Weight = 21.46 ± 0.84 grams

Table 5

Distribution of Activity 1.0 Hour Following I.V. Injection of ^{90}Y-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.27 ± 0.06	39.23 ± 2.45	49.77 ± 1.72
Heart	0.086 ± 0.01	5.80 ± 0.84	0.50 ± 0.09
Lung (2)	0.137 ± 0.01	12.11 ± 1.08	1.66 ± 0.17
Kidney (1)	0.120 ± 0.01	10.23 ± 1.30	1.15 ± 0.12
Liver	0.921 ± 0.05	12.12 ± 1.72	11.17 ± 1.66
Spleen	0.080 ± 0.01	9.27 ± 0.46	0.74 ± 0.07
Muscle	7.27 ± 0.32	0.78 ± 0.13	5.72 ± 1.05
Bone	2.73 ± 0.12	4.35 ± 0.39	11.89 ± 1.47
Skin	2.73 ± 0.12	2.12 ± 0.78	5.82 ± 2.24
GI Tract	2.22 ± 0.06	3.52 ± 1.12	4.22 ± 0.84
Urine	--	--	--
Feces	--	--	--
		TOTAL	94.85 ± 3.47

No. Mice = 5

Mean Weight = 18.17 grams ± 0.81 grams

Table 6

Distribution of Activity at 24 Hours Following I.V. Injection of ^{90}Y-2B8-MX-DTA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.517 ± 0.090	8.35 ± 2.547	12.83 ± 4.60
Heart	0.092 ± 0.005	2.63 ± 0.142	0.240 ± 0.006
Lung	0.141 ± 0.005	4.56 ± 0.393	0.644 ± 0.047
Kidney	0.138 ± 0.007	5.63 ± 0.222	0.779 ± 0.040
Liver	0.438 ± 0.098	5.22 ± 0.335	2.259 ± 0.399
Spleen	0.081 ± 0.003	4.23 ± 0.180	0.345 ± 0.011
Muscle	8.668 ± 0.514	0.976 ± 0.164	8.55 ± 1.945
Bone	3.246 ± 0.186	1.326 ± 0.102	4.289 ± 0.154

No. Mice = 3

Mean Weight = 21.671 ± 1.11 gram

Table 7

Distribution of Activity at 48 Hours Following I.V. Injection of ^{90}Y-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.33 ± 0.06	17.34 ± 2.0	23.03 ± 1.95
Heart	0.088 ± 0.01	3.56 ±0.31	0.31 ± 0.04
Lung (2)	0.139± 0.01	7.54 ± 0.88	1.05± 0.15
Kidney (1)	0.122 ± 0.01	6.53 ± 0.42	0.79 ± 0.01
Liver	0.968 ± 0.04	9.05 ± 1.70	8.92 ± 1.57
Spleen	0.087 ± 0.01	6.52 ±1.13	0.57 ± 0.07
Muscle	7.26 ± 0.36	1.05 ±0.18	8.01±1.17
Bone	2.86 ± 0.14	3.34 ± 0.42	9.53 ± 1.08
Skin	2.86 ± 0.14	4.13 ± 0.76	11.75 ± 1.82
GI Tract	2.84 ± 0.19	2.74 ± 0.34	3.80 ± 0.30
Urine	--	--	4.29
Feces	--	--	7.67
		TOTAL	79.72 ± 3.23

No. Mice = 5

Mean Weight = 19.07 ± 0.91 grams

Table 8

Distribution of Activity at 72 Hours Following I.V. Injection of ^{90}Y-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight	% ID/ Gram	% ID per Organ
Blood	1.35 ± 0.02	15.40 ±1.63	20.71 ± 2.13
Heart	0.088 ± 0.01	3.12 ± 0.24	0.28 ± 0.01
Lung (2)	0.142± 0.01	8.23 ± 1.05	1.17 ±0.20
Kidney (1)	0.123 ± 0.01	6.45 ± 0.57	0.79 ± 0.07
Liver	0.02 ± 0.06	8.39 ± 1.04	8.58 ± 1.31
Spleen	0.103 ± 0.01	5.90 ±1.19	0.59 ± 0.08
Muscle	7.68 ± 0.11	1.01 ± 0.15	7.73 ± 1.05
Bone	2.88 ± 0.05	3.20 ± 0.25	9.20 ± 0.61
Skin	2.88 ± 0.05	3.97 ± 0.49	11.42 ± 1.36
GI Tract	2.86 ± 0.18	2.90 ± 0.65	4.06 ± 0.93
Urine	--	--	3.00
Feces	--	--	11.08
		TOTAL	78.62 ± 2.63

No. Mice = 5

Mean Weight = 19.21 ± 0.27 grams

In a similar manner the other biological half-life values were assigned or calculated using the standard equation for calculating the $t_{1/2}$ for an exponential decay. Once these values had been determined, the variables for T_{ue}, T_{e1}, T_{e2}, A_1, A_2, and A, listed in Tables 9 and 10, were determined for each radiolabeled conjugate using the equations provided at the top of these tables (output variables). These values, as well as those shown in the subsequent tables, were calculated using a program written in the Symphony spreadsheet (Lotus Development Corp.) by Mr. Phillip Hagan, MS, Nuclear Medicine Service, VA Medical Center, La Jolla, CA 92161.

Table 9

INPUT VARIABLES

A0 = Administered doseTue = Effective uptake half-time
Tp = Physical half-life of radionuclide
Tu = Biologic uptake half-time
Tbl = Biological disappearance half-time of first component
Tb2 = Biological disappearance half-time of second component
fl = Fraction of A0 with biological half-time of Tb1
f2 = Fraction of A0 with biological half-time of Tb2
S = Mean Dose/Unit Cumulated Activity

OUTPUT VARIABLES

Tel = Effective disappearance half-time of first component
Te2 = Effective disappearance half-time of second component
Al = Cumulated activity of first component
A2 = Cumulated activity of second component
A = Total cumulated activity
Tue = Tu * Tp /(Tu + Tp) Al-1.44*f1*A0*Tel*(Tue/Tu)
Tel = Tbl * Tp /(Tbl + Tp) A2-1.44*f2*A0*Te2
Te2 = Tb2 * Tp /(Tb2 + Tp) A - Al + A2

Example: Al FOR LIVER - 1.44 * 11.000% * 1000 * 63.2 * 1.00 = 10007.5 microcuries cumulated activity
TABLE OF INPUT AND OUTPUT VALUES USED TO EVALUATE CUMULATED ACTIVITY (A)

	Tu (hr)	f1	f2	Tb1 (hr)	Tb2 (hr)	Tue (hr)	Tel (hr)	Te2 (hr)	Al (uCi-hr)	A2 (uCi-hr)	A (uCi-hr)
ADRENALS	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
BLAD CONTENTS	2.78E-04	1.000%	0.00%	4	0	2.78E-04	3.8	0.0	54.4	0.0	54
STOMACH CONTENTS	2.78E-04	6.650%	0.00%	1.5	0	2.78E-04	1.5	0.0	140.5	0.0	141
SM.INT. CONTENTS	2.78E-04	6.650%	0.00%	3.5	0	2.78E-04	3.3	0.0	318.6	0.0	319
ULI_CONTENTS	2.78E-04	6.650%	0.00%	4.5	0	2.78E-04	4.2	0.0	404.0	0.0	404
LLI_CONTENTS	2.78E-04	6.650%	0.00%	4.2	0	2.78E-04	4.0	0.0	378.6	0.0	379
KIDNEYS	2.78E-04	1.220%	0.00%	35	0	2.78E-04	23.0	0.0	404.8	0.0	405
LIVER	2.78E-04	11.000%	0.00%	1000	0	2.78E-04	63.2	0.0	10007.5	0.0	10008
LUNGS	2.78E-04	2.100%	1.20%	30	1000	2.78E-04	20.8	63.2	627.9	1091.7	1720
OTH TISS (TOTAL)	2.78E-04	0.000%									

	Tu (hr)	f1	f2	Tb1 (hr)	Tb2 (hr)	Tue (hr)	Te1 (hr)	Te2 (hr)	A1 (uCi-hr)	A2 (uCi-hr)	A (uCi-hr)
MUSCLE	2.78E-04	10.400%	0.00%	1000	0	2.78E-04	63.2	0.0	9461.7	0.0	9462
ADIPOSE	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
BLOOD	2.78E-04	58.400%	32.22%	15	1000	2.78E-04	12.3	63.2	10319.2	29313.1	39632
BRAIN	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
HEART	2.78E-04	0.510%	0.38%	57	1000	2.78E-04	30.9	63.2	226.9	345.7	573
PVARIES	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
PANCREAS	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
SKELETON (TOTAL)	2.78E-04	0.000%									
CORTICAL BONE	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
TRABECULAR BONE	2.78E-04	9.100%	6.30%	45	1000	2.78E-04	27.0	63.2	3536.8	5731.6	9268
NARROW (RED)	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
MARROW (YELLOW)	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
CARTILAGE	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
OTHER CONSTIT.	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
SKIN	2.78E-04	11.770%	0.00%	1000	0	2.78E-04	63.2	0.0	10708.1	0.0	10708
SPLEEN	2.78E-04	0.610%	0.40%	39	1000	2.78E-04	24.7	63.2	217.1	363.9	581
TESTES	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
THYROID	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	63.2	0.0	0.0	0.0	0
TOTAL BODY	2.78E-04	0.000%	0.00%	1000							

Table 10

INPUT VARIABLES

A0 = Administered dose
Tp = Physical half-life of radionuclide
Tu = Biologic uptake half-time
Tb1 = Biological disappearance half-time of first component
Tb2 = Biological disappearance half-time of second component
f1 = Fraction of A0 with biological half-time of Tb1
f2 = Fraction of A0 with biological half-time of Tb2
S = Mean Dose/Unit Cumulated Activity

OUTPUT VARIABLES

Tue = Effective uptake half-time
Tel = Effective disappearance half-time of first component
Te2 = Effective disappearance half-time of second component
Al = Cumulated activity of first component
A2 = Cumulated activity of second component
A = Total cumulated activity
Tue = Tu * Tp /(Tu + Tp) Al-1.44*f1*A0*Tel*(Tue/Tu)
Tel = Tbl * Tp /(Tbl + Tp) A2-1.44*f2*A0*Te2
Te2 = Tb2 * Tp /(Tb2 + Tp) A - Al + A2

Example: Al FOR LIVER - 1.44 * 9.000% * 1000 * 60.2 * 1.00 = 7795.5 microcuries cumulated activity
TABLE OF INPUT AND OUTPUT VALUES USED TO EVALUATE CUMULATED ACTIVITY (A)

	Tu (hr)	f1	f2	Tb1 (hr)	Tb2 (hr)	Tue (hr)	Tel (hr)	Te2 (hr)	Al (uCi-hr)	A2 (uCi-hr)	A (uCi-hr)
ADRENALS	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
BLAD CONTENTS	2.78E-04	1.000%	0.00%	4	0	2.78E-04	3.8	0.0	54.2	0.0	54
STOMACH CONTENTS	2.78E-04	4.220%	0.00%	1.5	0	2.78E-04	1.5	0.0	89.1	0.0	89
SM.INT. CONTENTS	2.78E-04	4.220%	0.00%	3.5	0	2.78E-04	3.3	0.0	201.7	0.0	202
ULI_CONTENTS	2.78E-04	4.220%	0.00%	4.5	0	2.78E-04	4.2	0.0	255.5	0.0	255
LLI_CONTENTS	2.78E-04	4.220%	0.00%	4.2	0	2.78E-04	3.9	0.0	239.5	0.0	240
KIDNEY	2.78E-04	1.150%	0.87%	70	1000	2.78E-04	33.4	60.2	553.6	753.6	1307
LIVER	2.78E-04	9.000%	0.00%	1000	0	2.78E-04	60.2	0.0	7795.5	0.0	7795
LUNGS	2.78E-04	1.200%	0.00%	1000	0	2.78E-04	60.2	0.0	1039.4	0.0	1039
OTH TISS (TOTAL)	2.78E-04	0.000%									
MUSCLE	2.78E-04	8.720%	0.00%	1000	0	2.78E-04	60.2	0.0	7552.9	0.0	7553
ADIPOSE	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
BLOOD	2.78E-04	49.770%	25.90%	13	1000	2.78E-04	10.8	60.2	7743.9	22433.7	30178

	Tu (hr)	f1	f2	Tb1 (hr)	Tb2 (hr)	Tue (hr)	Te1 (hr)	Te2 (hr)	A1 (uCi-hr)	A2 (uCi-hr)	A (uCi-hr)
BRAIN	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
HEART	2.78E-04	0.500%	0.36%	51	1000	2.78E-04	28.4	60.2	204.4	311.8	516
OVARIES	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
PANCREAS	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
SKELETON (TOTAL)	2.78E-04	0.000%									
CORTICAL BONE	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
TRABECULAR BONE	2.78E-04	11.890%	9.28%	67	1000	2.78E-04	32.7	60.2	5604.4	8038.0	13642
MARROW (RED)	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
MARROW (YELLOW)	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
CARTILAGE	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
OTHER CONSTIT.	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
SKIN	2.78E-04	15.600%	0.00%	1000	0	2.78E-04	60.2	0.0	13512.1	0.0	13512
SPLEEN	2.78E-04	0.740%	0.56%	60	1000	2.78E-04	31.0	60.2	330.0	485.1	815
TESTES	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
THYROID	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0
TOTAL BODY	2.78E-04	0.000%	0.00%	1000	0	2.78E-04	60.2	0.0	0.0	0.0	0

Using the Total Cumulated Activity (A) values from Tables 9 and 10, and the S values provided from MIRD Pamphlet Number 11 (Tables 11 and 12, and 13 and 14), the radiation absorbed dose estimates were determined for each of the radiolabeled conjugates for the listed tissues (Tables 15, 16, 17 and 18). In determining the summary radiation dose estimates for the indium-labeled conjugate provided in Table 19, the self-dose of a given organ was summed with the absorbed dose produced by activity in adjacent organs or tissues. However, in calculating the radiation dose estimate values attributed to the yttrium-labeled conjugate (Table 20), certain of the values are absent for the listed tissues (e.g. adrenals). This is due to the shorter path length of the released 13 particle, relative to the path-length of the emitted g particle, hence providing a negligible activity contribution from adjacent tissues, and to the absence of primary biodistribution data for these tissues.

Table 11

S. ABSORBED DOSE PER UNIT CUMULATED ACTIVITY, (RAD/UCI-H)
INDIUM-[111] HALF-LIFE 67.44 HOURS

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue (Muscle)
			Stomach Contents	Si Contents	Uli Contents	Lli Contents				
ADRENALS	7.4E-03	5.7E-07	7.3E-06	4.4E-06	2.8E-06	1.3E-06	3.4E-05	1.5E-05	7.6E-06	4.8E-06
BLADDER WALL	3.6E-07	4.5E-04	7.5E-07	8.0E-06	6.4E-06	2.0E-05	9.3E-07	5.2E-07	1.5E-07	5.5E-06
BONE	5.2E-06	2.3E-06	2.3E-06	3.2E-06	2.9E-06	4.2E-06	3.7E-06	2.9E-06	3.8E-06	3.2E-06
GI (STOM WALL)	8.8E-06	8.5E-07	3.4E-04	1.1E-05	1.2E-05	5.4E-06	1.0E-05	5.8E-06	5.7E-06	4.3E-06
GI (SI)	2.5E-06	8.6E-06	7.9E-06	2.1E-04	5.4E-05	3.0E-05	8.6E-06	5.0E-06	6.1E-07	4.8E-06
GI (ULI WALL)	2.8E-06	6.9E-06	1.1E-05	8.3E-05	3.3E-04	1.4E-05	8.6E-06	7.5E-06	7.4E-07	5.0E-06
GI (LLI WALL)	7.1E-07	2.2E-05	3.8E-06	2.4E-05	9.5E-06	4.7E-04	2.5E-06	7.3E-07	3.0E-07	5.2E-06

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue (Muscle)
			Stomach Contents	Si Contents	Uli Contents	Lli Contents				
KIDNEYS	3.7E-05	8.5E-07	1.1E-05	9.2E-06	8.3E-06	2.8E-06	5.2E-04	1.2E-05	2.7E-06	4.4E-06
LIVER	1.5E-05	6.3E-07	5.9E-06	5.6E-06	7.8E-06	8.4E-07	1.2E-05	1.3E-04	7.7E-06	3.4E-06
LUNGS	7.6E-06	8.2E-08	5.2E-06	7.5E-07	8.3E-07	2.6E-07	2.5E-06	7.8E-06	1.4E-04	4.2E-06
MARROW (RED)	9.4E-06	5.3E-06	4.0E-06	1.1E-05	9.1E-06	1.3E-05	9.6E-06	4.1E-06	4.8E-06	5.3E-06
OTH TISS (MUSC)	4.8E-06	5.5E-06	4.3E-06	4.8E-06	4.5E-06	5.2E-06	4.4E-06	3.4E-06	4.4E-06	7.5E-06
OVARIES	1.8E-06	2.3E-05	1.3E-06	3.3E-05	3.7E-05	6.4E-05	3.6E-06	1.4E-06	3.6E-07	6.3E-06
PANCREAS	2.6E-05	8.6E-07	5.7E-05	6.1E-06	7.1E-06	2.1E-06	2.0E-05	1.2E-05	7.7E-06	5.7E-06
SKIN	1.8E-06	1.7E-06	1.4E-06	1.4E-06	1.4E-06	1.6E-06	1.8E-06	1.6E-06	1.8E-06	2.5E-06
SPLEEN	2.0E-05	7.6E-07	3.1E-05	4.6E-06	4.2E-06	2.4E-06	2.8E-05	2.8E-06	7.1E-06	4.6E-06
TESTES	1.4E-07	1.4E-05	1.8E-07	1.0E-06	9.8E-07	5.9E-06	3.4E-07	2.5E-07	3.9E-08	3.6E-06
THYROID	4.7E-07	1.2E-08	3.5E-07	6.9E-08	7.5E-08	2.7E-08	2.0E-07	6.2E-07	2.6E-06	4.3E-06

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue (Muscle)
			Stomach Contents	Si Contents	Uli Contents	Lli Contents				
UTERUS (NONGRVD)	5.8E-06	4.9E-05	2.4E-06	2.9E-05	1.5E-05	2.1E-05	3.1E-06	1.2E-06	2.8E-07	7.4E-06
TOTAL BODY	6.6E-06	6.2E-06	6.1E-06	7.3E-06	6.8E-06	6.9E-06	6.6E-06	6.6E-06	5.9E-06	5.6E-06

REFERENCE - MIRD PAMPHLET NO. 11, PAGE 164

Table 12
S. ABSORBED DOSE PER UNIT CUMULATED ACTIVITY, (RAD/UCI-H)
INDIUM-[111] HALF-LIFE 67.44 HOURS

Target Organs	SOURCE ORGANS									
	Ovaries	Pancreas	Skeleton			Skin	Spleen	Testes	Thyroid	Total Body
			R Marrow	Cort Bone	TRA Bone					
ADRENALS	1.1E-06	2.6E-05	7.9E-06	3.9E-06	3.9E-06	2.4E-06	2.0E-05	1.4E-07	4.7E-07	7.0E-06
BLADDER WALL	2.1E-05	4.7E-07	2.4E-06	1.5E-06	1.5E-06	1.6E-06	4.7E-07	1.5E-05	1.2E-08	6.9E-06
BONE	3-8E-06	3.6E-06	1.2E-05	3.0E-05	2.6E-05	2.9E-06	2.9E-06	2.4E-06	2.6E-06	6.9E-06
GI (STOM WALL)	2.4E-06	5.9E-05	3.2E-06	1.7E-06	1.1E-06	1.7E-06	3.0E-05	1.5E-07	1.5E-07	7.1E-06
GI (SI)	3.8E-05	5.5E-06	7.9E-06	2.3E-06	2.3E-06	1.5E-06	4.2E-06	1.2E-06	4.2E-08	7.5E-06
GI (ULI WALL)	3.7E-05	6.6E-06	6.4E-06	2.2E-06	2.2E-06	1.4E-06	3.8E-06	1.1E-06	3.3E-08	7.0E-06
GI (LLI WALL)	4.8E-05	1.7E-06	9.0E-06	3.2E-06	3.2E-06	1.5E-06	1.9E-06	8.3E-06	2.2E-08	6.7E-06
KIDNEYS	2.9E-06	1.9E-05	6.8E-06	2.7E-06	2.7E-06	2.0E-06	2.8E-05	1.7E-07	1.2E-07	6.6E-06
LIVER	1.7E-06	1.3E-05	2.9E.06	2.0E-06	2.0E-06	1.7E-06	3.0E-06	1.2E-07	3.5E-07	6.5E-06
LUNGS	2.2E-07	7.6E-06	3.7E-06	3.0E-06	3.0E-06	1.9E-06	6.9E-06	3.4E-08	2.9E-06	5.9E-06
MARROW (RED)	1.3E-05	6.8E-06	7.5E-05	1.3E-05	2.6E-05	2.7E-06	4.4E-06	1.9E-06	2.9E-06	7.7E-06
OTH TISS (MUSC)	6.3E-06	5.7E-06	3.8E-06	3.2E-06	3.2E-06	2.5E-06	4.6E-06	.3.6E-06	4.3E-06	5.6E-06

Target Organs	SOURCE ORGANS Ovaries	Pancreas	Skeleton R Marrow	Skeleton Cort Bone	Skeleton TRA Bone	Skin	Spleen	Testes	Thyroid	Total Body
OVARIES	1.0E-02	1.0E-06	7.7E-06	2.2E-06	2.2E-06	1.4E-06	1.7E-06	0.0E+00	2.5E-08	7.0E-06
PANCREAS	1.5E-06	1.6E-03	4.9E-06	3.1E-06	3.1E-06	1.7E-06	6.1E-05	2.1E-07	3.0E-07	7.8E-06
SKIN	1.4E-06	1.3E-06	2.0E-06	2.3E-06	2.3E-06	3.7E-05	1.5E-06	4.9E-06	2.5E-06	3.7E-06
SPLEEN	1.6E-06	6.2E-05	2.7E-06	2.0E-06	2.0E-06	1.7E-06	9.1E-04	8.9E-08	3.5E-07	6.8E-06
TESTES	0.0E+00	2.1E-07	1.0E-06	1.9E-06	1.9E-06	3.4E-06	2.0E-07	3.6E-03	3.3E-09	4.9E-06
THYROID	2.5E-08	4.5E-07	2.2E-06	2.8E-06	2.8E-06	2.4E-06	3.4E-07	3.3E-09	5.8E-03	5.2E-06
UTERUS (NONGRVO)	6.5E-05	1.9E-06	6.7E-06	1.8E-06	1.8E-06	1.2E-06	1.2E-06	0.0E+00	2.4E-08	7.8E-06
TOTAL BODY	7.7E-06	7.5E-06	6.4E-06	5.9E-06	5.9E-06	3.8E-06	6.6E-06	5.6E-06	5.3E-06	5.8E-06

REFERENCE - MIRD PAMPHLET NO. 11. PAGE 165

Table 13

S. ABSORBED DOSE PER UNIT CUMULATED ACTIVITY, (RAD/UCI-H)

YTTRIUM-[90] HALF-LIFE 64 HOURS

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue (Muscle)
			Stomach Contents	SI Contents	ULI Contents	LLI Contents				
ADRENALS	1.4E-01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BLADDER WALL	0.0	5.0E-03	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BONE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI (STOM WALL)	0.0	0.0	4.0E-03	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI (SI)	0.0	0.0	0.0	2.5E-03	0.0	0.0	0.0	0.0	0.0	0.0
GI (ULI WALL)	0.0	0.0	0.0	0.0	4.5E-03	0.0	0.0	0.0	0.0	0.0
GI (LLI WALL)	0.0	0.0	0.0	0.0	0.0	7.4E-03	0.0	0.0	0.0	0.0
KIDNEYS	0.0	0.0	0.0	0.0	0.0	0.0	6.4E-03	0.0	0.0	0.0
LIVER	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1E-03	0.0	0.0
LUNGS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0E-03	0.0
MARROW (RED)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue (Muscle)
			Stomach Contents	SI Contents	ULI Contents	LLI Contents				
OTH TISS (MUSC)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.1E-05
OVARIES	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PANCREAS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SKIN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SPLEEN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TESTES	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
THYROID	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UTERUS (NONGRVD)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TOTAL BODY	2.8E-05	3.2E-06	8.5E-05	2.3E-05	1.4E-05	1.7E-05	2.8E-05	2.8E-05	2.8E-05	2.8E-05

REFERENCE - MIRD PAMPHLET NO. 11, PAGE 144

Table 14

S. ABSORBED DOSE PER UNIT CUMULATED ACTIVITY, (RAD/UCI-H)

YTTRIUM-[90] HALF-LIFE 64 HOURS

Target Organs	SOURCE ORGANS									
	Ovaries	Pancreas	Skeleton			Skin	Spleen	Testes	Thyroid	Total Body
			R Marrow	Cort Bone	TRA Bone					
ADRENALS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
BLADDER WALL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
BONE	0.0	0.0	1.1E-04	4.0E-04	2.3E-04	0.0	0.0	0.0	0.0	2.8E-05
GI (STOM WALL)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
GI (SI)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
GI (ULI WALL)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
GI (LLI WALL)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
KIDNEYS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
LIVER	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
LUNGS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
MARROW (RED)	0.0	0.0	8.6E-04	3.3E-05	5.7E-04	0.0	0.0	0.0	0.0	2.8E-05
OTH TISS (MUSC)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05

Target Organs	SOURCE ORGANS Ovaries	Pancreas	Skeleton R Marrow	Skeleton Cort Bone	Skeleton TRA Bone	Skin	Spleen	Testes	Thyroid	Total Body
OVARIES	1.8E-01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
PANCREAS	0.0	2.0E-02	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
SKIN	0.0	0.0	0.0	0.0	0.0	7.6E-04	0.0	0.0	0.0	2.8E-05
SPLEEN	0.0	0.0	0.0	0.0	0.0	0.0	1.1E-02	0.0	0.0	2.8E-05
TESTES	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.7E-02	0.0	2.8E-05
THYROID	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.9E-02	2.8E-05
UTERUS (NONGRVO)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8E-05
TOTAL BODY	2.8E-05	2.8E-05	2.8E-05	2.8E-05	2.8E-05	2.8E-05	2.8E-05	2.8E-05	2.8E-05	2.8E-05

REFERENCE - MIRD PAMPHLET NO. 11. PAGE 145

Table 15
RADIATION ABSORBED DOSE (RAD = A · S)
INDIUM-[111] HALF-LIFE 67.44 HOURS

Target Organs	SOURCE ORGANS Adrenals	Bladder Contents	Intestinal Tract Stomach Contents	SI Contents	ULI Contents	LLI Contents	Kidneys	Liver	Lungs	Other Tissue
ADRENALS	0.0E+00	3.1E-05	1.0E-03	1.4E-03	1.1E-03	4.9E-04	1.4E-02	1.5E-01	1.3E-02	2.4E-01
BLADDER WALL	0.0E+00	2.4E-02	1.1E-04	2.5E-03	2.6E-03	7.6E-03	3.8E-04	5.2E-03	2.6E-04	2.7E-01
GI (STOM WALL)	0.0E+00	4.6E-05	4.8E-02	3.5E-03	2.2E-02	2.0E-03	4.0E-03	5.8E-02	9.8E-03	2.1E-01
GI (SI)	0.0E+00	4.7E-04	1.1E-03	6.7E-03	2.2E-02	1.1E-02	3.5E-03	5.0E-02	1.0E-03	2.4E-01
GI (ULI WALL)	0.0E+00	3.8E-04	1.5E-03	2.6E-02	1.3E-01	5.3E-03	3.5E-03	7.5E-02	1.3E-03	2.5E-01
GI (LLI WALL)	0.0E+00	1.2E-03	5.3E-04	7.6E-03	3.8E-03	1.8E-01	1.0E-03	7.3E-03	5.2E-04	2.6E-01
KIDNEYS	0.0E+00	4.6E-05	1.5E-03	2.9E-03	3.4E-03	1.1E-03	2.1E-01	1.2E-01	4.6E-03	2.2E-01
LIVER	0.0E+00	3.4E-05	8.3E-04	1.8E-03	3.2E-03	3.2E-04	4.9E-03	1.3E+00	1.3E-02	1.7E-01
LUNGS	0.0E+00	4.5E-06	7.3E-04	2.4E-04	3.4E-04	9.8E-05	1.0E-03	7.8E-02	2.4E-01	2.1E-01
OTHER TISSUES										
MUSCLE	0.0E+00	3.0E-04	6.0E-04	1.5E-03	1.8E-03	2.0E-03	1.8E-03	3.4E-02	7.6E-03	3.7E-01

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue
			Stomach Contents	SI Contents	ULI Contents	LLI Contents				
ADIPOSE	0.0E+00	3.0E-04	6.0E-04	1.5E-03	1.8E-03	2.0E-03	1.8E-03	3.4E-02	7.6E-03	3.7E-01
BLOOD	0.0E+00	3.0E-04	6.0E-04	1.5E-03	1.8E-03	2.0E-03	1.8E-03	3.4E-02	7.6E-03	3.7E-01
BRAIN	0.0E+00	3.0E-04	6.0E-04	1.5E-03	1.8E-03	2.0E-03	1.8E-03	3.4E-02	7.6E-03	3.7E-01
HEART	0.0E+00	4.1E-05	4.4E-03	1.5E-03	1.7E-03	9.1E-04	1.1E-02	2.8E-02	1.2E-02	3.7E-01
OVARIES	0.0E+00	1.3E-03	1.8E-04	1.1E-02	1.5E-02	2.4E-02	1.5E-03	1.4E-02	6.2E-04	2.8E-01
PANCREAS	0.0E+00	4.7E-05	8.0E-03	1.9E-03	2.9E-03	8.0E-04	8.1E-03	1.2E-01	1.3E-02	1.2E-01
SKELETON										
CORTICAL BONE	0.0E+00	1.3E-04	3.2E-04	1.0E-03	1.2E-03	1.6E-03	1.5E-03	2.9E-02	6.5E-03	1.6E-01
TRABECULAR BONE	0.0E+00	1.3E-04	3.2E-04	1.0E-03	1.2E-03	1.6E-03	1.5E-03	2.9E-02	6.5E-03	1.6E-01
MARROW (RED)	0.0E+00	2.9E-04	5.6E-04	3.5E-03	3.7E-03	4.6E-03	3.9E-03	4.1E-02	8.3E-03	2.6E-01
MARROW (YELLOW)	0.0E+00	2.9E-04	5.6E-04	3.5E-03	3.7E-03	4.9E-03	3.9E-03	4.1E-02	8.3E-03	2.6E-01
CARTILAGE	0.0E+00	1.3E-04	3.2E-04	1.0E-03	1.2E-03	1.6E-03	1.5E-03	2.9E-02	6.5E-03	1.6E-01
OTHER CONSTIT.	0.0E+00	1.3E-04	3.2E-04	1.0E-03	1.2E-03	1.6E-03	1.5E-03	2.9E-02	6.5E-03	1.6E-01
SKIN	0.0E+00	9.2E-05	2.0E-04	4.5E-04	5.7E-04	6.1E-04	7.3E-04	1.6E-02	3.1E-03	1.2E-01

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue
			Stomach Contents	SI Contents	ULI Contents	LLI Contents				
SPLEEN	0.0E+00	4.1E-05	4.4E-03	1.5E-03	1.7E-03	9.1E-04	1.1E-02	2.8E-02	1.2E-02	2.3E-01
TESTES	0.0E+00	7.6E-04	2.5E-05	3.2E-04	4.0E-04	2.2E-03	1.4E-04	2.5E-03	6.7E-05	1.8E-01
THYROID	0.0E+00	6.5E-07	4.9E-05	2.2E-05	3.0E-05	1.0E-05	8.1E-05	6.2E-03	4.5E-03	2.1E-01
UTERUS (NONGRVD)	0.0E+00	3.4E-04	8.6E-04	2.3E-03	2.7E-03	2.6E-03	2.7E-03	6.6E.02	1.0E-02	3.7E-01
TOTAL BODY	0.0E+00	2.7E.03	3.4E.04	9.2E-03	6.1E-03	8.0E.03	1.3E-03	1.2E-02	4.8E-04	2.8E-01

Table 16

RADIATION ABSORBED DOSE (RAD = A · S)
INDIUM-[111] HALF-LIFE 67.44 HOURS

Target Organs	SOURCE ORGANS									
	Ovaries	Pancreas	Skeleton			Skin	Spleen	Testes	Thyroid	Total Body
			R Marrow	Cort Bone	TRA Bone					
ADRENALS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.6E-02	2.6E-02	1.2E-02	0.0E+00	0.0E+00	0.0E+00
BLADDER WALL	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.4E-02	1.7E-02	2.7E-04	0.0E+00	0.0E+00	0.0E+00
GI (STOM WALL)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.6E-02	1.8E-02	1.7E-02	0.0E+00	0.0E+00	0.0E+00
GI (SI)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.1E-02	1.6E-02	2.4E-03	0.0E+00	0.0E+00	0.0E+00
GI (ULI WALL)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.0E-02	1.5E-02	2.2E-03	0.0E+00	0.0E+00	0.0E+00

Target Organs	SOURCE ORGANS Ovaries	Pancreas	Skeleton R Marrow	Skeleton Cort Bone	Skeleton TRA Bone	Skin	Spleen	Testes	Thyroid	Total Body
GI (LLI WALL)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.0E-02	1.6E-02	1.1E-03	0.0E+00	0.0E+00	0.0E+00
KIDNEYS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.5E-02	2.1E-02	1.6E-02	0.0E+00	0.0E+00	0.0E+00
LIVER	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.9E-02	1.8E-02	1.7E-03	0.0E+00	0.0E+00	0.0E+00
LUNGS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.8E-02	2.0E-02	4.0E-03	0.0E+00	0.0E+00	0.0E+00
OTHER TISSUES										
MUSCLE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.0E-02	2.7E-02	2.7E-03	0.0E+00	0.0E+00	0.0E+00
ADIPOSE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.0E-02	2.7E-02	2.7E-03	0.0E+00	0.0E+00	0.0E+00
BLOOD	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.0E-02	2.7E-02	2.7E-03	0.0E+00	0.0E+00	0.0E+00
BRAIN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.0E-02	2.7E-02	2.7E-03	0.0E+00	0.0E+00	0.0E+00

Target Organs	SOURCE ORGANS									
	Ovaries	Pancreas	Skeleton			Skin	Spleen	Testes	Thyroid	Total Body
			R Marrow	Cort Bone	TRA Bone					
HEART	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.0E-02	2.7E-02	2.7E-03	0.0E+00	0.0E+00	0.0E+00
OVARIES	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.0E-02	1.5E-02	9.9E-04	0.0E+00	0.0E+00	0.0E+00
PANCREAS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.9E-02	1.8E-02	3.5E-02	0.0E+00	0.0E+00	0.0E+00
SKELETON										
CORTICAL BONE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.4E-01	3.1E-02	1.7E-03	0.0E+00	0.0E+00	0.0E+00
TRABECULAR BONE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.4E-01	3.1E-02	1.7E-03	0.0E+00	0.0E+00	0.0E+00
MARROW (RED)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.4E-01	2.9E-02	2.6E-03	0.0E+00	0.0E+00	0.0E+00
MARROW (YELLOW)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.4E-01	2.9E-02	2.6E-03	0.0E+00	0.0E+00	0.0E+00
CARTILAGE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.4E-01	3.1E-02	1.7E-03	0.0E+00	0.0E+00	0.0E+00

Target Organs	SOURCE ORGANS									
	Ovaries	Pancreas	Skeleton			Skin	Spleen	Testes	Thyroid	Total Body
			R Marrow	Cort Bone	TRA Bone					
OTHER CONSTIT.	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.4E-01	3.1E-02	1.7E-03	0.0E+00	0.0E+00	0.0E+00
SKIN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.1E-02	4.0E-01	8.7E-04	0.0E+00	0.0E+00	0.0E+00
SPLEEN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.9E-02	1.8E-02	5.3E-01	0.0E+00	0.0E+00	0.0E+00
TESTES	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.8E-02	3.6E-02	1.2E-04	0.0E+00	0.0E+00	0.0E+00
THYROID	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.6E-02	2.6E-02	2.0E-04	0.0E+00	0.0E+00	0.0E+00
UTERUS (NONGRVO)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.7E-02	0.0E+00	7.0E-04	0.0E+00	0.0E+00	0.0E+00
TOTAL BODY	0.0E+00	0.0E+00	0.0E+00	0.0E+00	5.5E-02	4.1E-02	3.8E-03	0.0E+00	0.0E+00	0.0E+00

Table 17

RADIATION ABSORBED DOSE (RAD = A · S)
YTTRIUM-[90] HALF-LIFE 64 HOURS

Target Organs	SOURCE ORGANS: Adrenals	Bladder Contents	Intestinal Tract: Stomach Contents	SI Contents	ULI Contents	LLI Contents	Kidneys	Liver	Lungs	Other Tissue
ADRENALS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
BLADDER WALL	0.0E+00	2.7E-01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
GI (STOM WALL)	0.0E+00	0.0E+00	3.6E-01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
GI (SI)	0.0E+00	0.0E+00	0.0E+00	5.0E-01	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
GI (ULI WALL)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.1E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
GI (LLI WALL)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.8E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue
			Stomach Contents	SI Contents	ULI Contents	LLI Contents				
KIDNEYS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	8.4E+00	0.0E+00	0.0E+00	0.0E+00
LIVER	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	8.6E+00	0.0E+00	0.0E+00
LUNGS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.1E+00	0.0E+00
OTHER TISSUES										
MUSCLE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.7E+00
ADIPOSE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.7E+00
BLOOD	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.7E+00
BRAIN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.7E+00
HEART	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.7E+00

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue
			Stomach Contents	SI Contents	ULI Contents	LLI Contents				
OVARIES	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
PANCREAS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
SKELETON										
CORTICAL BONE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
TRABECULAR BONE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
MARROW (RED)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
MARROW (YELLOW)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
CARTILAGE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
OTHER CONSTIT.	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Target Organs	SOURCE ORGANS									
	Adrenals	Bladder Contents	Intestinal Tract				Kidneys	Liver	Lungs	Other Tissue
			Stomach Contents	SI Contents	ULI Contents	LLI Contents				
SKIN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
SPLEEN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
TESTES	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
THYROID	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
UTERUS (NONGRVD)	0.0E+00	1.7E-04	7.6E-03	4.6E-03	3.6E-03	4.1E-03	3.7E-02	2.2E-01	2.9E-02	0.0E+00
TOTAL BODY	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.1E+00

Table 18

RADIATION ABSORBED DOSE (RAD = A • S)
YTTRIUM-[90] HALF-LIFE 64.00 HOURS

	SOURCE ORGANS									
Target Organs	Ovaries	Pancreas	Skeleton			Skin	Spleen	Testes	Thyroid	Total Body
			R Marrow	Cort Bone	TRA Bone					
ADRENALS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
BLADDER WALL	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
GI (STOM WALL)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
GI (SI)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
GI (ULI WALL)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
GI (LLI WALL)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
KIDNEYS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
LIVER	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
LUNGS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
OTHER TISSUES										

Target Organs	SOURCE ORGANS Ovaries	Pancreas	Skeleton R Marrow	Skeleton Cort Bone	Skeleton TRA Bone	Skin	Spleen	Testes	Thyroid	Total Body
MUSCLE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
ADIPOSE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
BLOOD	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
BRAIN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
HEART	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
OVARIES	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
PANCREAS	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
SKELETON										
CORTICAL BONE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.1E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
TRABECULAR BONE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.1E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
MARROW (RED)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	7.8E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
MARROW (YELLOW)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	7.8E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
CARTILAGE	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.1E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
OTHER CONSTIT.	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.1E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Target Organs	SOURCE ORGANS									
	Ovaries	Pancreas	Skeleton			Skin	Spleen	Testes	Thyroid	Total Body
			R Marrow	Cort Bone	TRA Bone					
SKIN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.0E+01	0.0E+00	0.0E+00	0.0E+00	0.0E+00
SPLEEN	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	9.0E+00	0.0E+00	0.0E+00	0.0E+00
TESTES	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
THYROID	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
UTERUS (NONGRVO)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
TOTAL BODY	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.8E-01	3.8E+01	2.3E-02	0.0E+00	0.0E+00	0.0E+00

Table 19

Radiation Dosimetry Estimates Resulting from the Administration of Indium-[111] Labeled 2B8-MX Uniformly Distributed in Standard Man(70Kg) and Based on Animal Distribution Data Over 72 Hours after Injection

AMOUNT OF ACTIVITY =		1000 MICROCURIES/PATIENT DOSE	
	RADS		RADS
ADRENALS	0.493	OVARIES	0.387
BLADDER WALL	0.348	PANCREAS	0.362
STOMACH WALL	0.412	SKELETON	
SMALL INTESTINE	0.434	CORTICAL BONE	0.474
UL INTEST. WALL	0.533	TRABECULAR BONE	0.474
LL INTEST. WALL	0.505	MARROW (RED)	0.602
KIDNEYS	0.625	MARROW (YELLOW)	0.602
LIVER	1.533	CARTILAGE	0.474
LUNGS	0.582	OTHER CONSTIT.	0.474
OTHER TISSUES		SKIN	0.564
MUSCLE		SPLEEN	0.854
ADIPOSE		TESTES	0.239
BLOOD		THYROID	0.276
BRAIN		UTERUS (NONGRVD)	0.473
HEART		TOTAL BODY	0.417

Ref: A Schema for Absorbed-dose Calculation for Biologically Distributed Radionuclides, MIRD J. of Nucl. Med./Suppl. #1, 2/68

Calculations Performed Using a Spreadsheet Template in Symphony (Lotus Development Corporation) and Created by

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Table 20

Radiation Dosimetry Estimates Resulting from the Administration of Yttrium-[90] Labeled 2B8-MX Uniformly Distributed in Standard Man(70 Kg) and Based on Animal Distribution Data Over 72 Hours After Injection

AMOUNT OF ACTIVITY =		1000 MICROCURIES/PATIENT DOSE	
	RADS		RADS
ADRENALS	0.000	OVARIES	0.000
BLADDER WALL	0.271	PANCREAS	0.000
STOMACH WALL	0.356	SKELETON	
SMALL INTESTINE	0.504	CORTICAL BONE	3.138
UL INTEST. WALL	1.150	TRABECULAR BONE	3.138
LL INTEST. WALL	1.772	MARROW (RED)	7.776
KIDNEYS	8.366	MARROW (YELLOW)	7.776
LIVER	8.575	CARTILAGE	3.138
LUNGS	2.079	OTHER CONSTIT.	3.138
OTHER TISSUES		SKIN	10.269
MUSCLE	2.716	SPLEEN	8.965
ADIPOSE	2.716	TESTES	0.000
BLOOD	2.716	THYROID	0.000
BRAIN	2.716	UTERUS (NONGRVD)	0.304
HEART	2.176	TOTAL BODY	1.854

Ref: A Schema for Absorbed-dose Calculation for Biologically Distributed Radionuclides, MIRD J. of Nucl. Med./Suppl. #1, 2/68

Calculations Performed Using a Spreadsheet Template in Symphony (Lotus Development Corporation) and Created by

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1. II. Results

A. *In Vitro* Studies With 2B8 and 2B8-MX-DTPA

1. Production and Characterization of the Anti-CD20 Antibody 2B8

A total of nine fusions resulted in three hybridomas producing antibodies which effectively competed with radiolabeled Coulter B1 antibody. In each case, the hybridoma was expanded into a 24 well plate. The first two antibodies isolated from fusions 3 and 4, were isotyped and both identified as IgM. The third antibody, produced in fusion 5 and designated 2B8, was determined to be an IgGl kappa isotype and was selected for continuation studies. Clone 2B8.Hll was expanded and placed in long term storage in liquid nitrogen. Clone 2B8.Hll was subcloned to produce clone 2B8.Hll.G3 and again to produce clone 2B8.Hll.G3.G9. This clone was expanded for further study and the antibody was purified by protein A affinity chromatography.

Competition assays using unlabeled 2B8, Bl and Leu 16 and radiolabeled Coulter B1 demonstrated that 2B8 was able to inhibit Bl binding to CD20 more effectively than equal concentrations of either Bl or Leu 16 (Fig. 1). Similar results were obtained (data not shown) in a competition study using FITC-conjugated 2B8, native Bl and the irrelevant antibodies UPC-10 and S-003 (IgG 2a and 1 isotypes, respectively).

Direct binding to cellular CD20 antigen by 2B8 and Bl antibodies was compared by FACS analysis using CD20-positive SB cells and CD20-negative HSB cells. The results shown in Figure 2 indicate that for comparable amounts of antibody, more 2B8 than Bl was bound to the SB cells. No significant binding to SB cells was observed with the irrelevant antibodies. Only background fluorescence was observed with any reagent used with HSB cells. These results confirm the specificity of interaction of 2B8 with the CD20 antigen and suggest that 2B8 may have higher affinity for the cell-surface antigen than Bl.

To determine the apparent affinity of 2B8, purified antibody was radiolabeled with ^{125}I and increasing concentrations of the labeled antibody were incubated with antigen-positive SB cells; cell-associated radioactivity was determined following a 1 hour incubation period (Fig. 3). The results suggest that the 2B8 antibody binds to the CD20 antigen with an apparent affinity constant of 4.3 X 10^{-9} M.

Flow cytometry studies with human normal peripheral blood lymphocytes indicated that 2B8 was specific for B-cells and did not react with other types of lymphocytes (e.g. T-cells, monocytes, macrophages). FITC-labeled 2B8 was compared to B1-FITC and Leu 16-FITC using the same population of human lymphocytes. The results shown in Table 21 indicate that 2B8 reacted with approximately 14 percent of the peripheral blood lymphocytes versus approximately 12 percent for Leu 16 and 11 percent for Bl. The lymphocyte population based on another B lymphocyte marker (CD-19) was between 11 and 14 percent. Finally, when human peripheral blood lymphocytes were incubated with 2B8 and either B1 or Leu 16 and then counterstained with the CD19 marker (Becton/Dickinson) the double staining population of B lymphocytes was 9 percent with 2B8, and 10 percent with either Bl or Leu 16. These results confirm the similarity of these reagents.

Table 21

Comparison of Binding of 2B8 to Human Peripheral Blood Lymphocytes with other B- and T-Lymphocyte Specific Reagents

Antibody Marker	
A. **Single Staining:**	**Percent of CD45 Gated Lymphocytes**
None (autofluorescence)	0
B1-FITC (Coulter Immunology, (IgG2a,k)	11
Leu 16-FITC (Becton Dickinson, IgGl,k)	12
2B8-FITC (EDEC, IgGl,k)	14
B72.3-FITC (IgGl,k irrelevant control)	4
anti-CD4-FITC (Coulter Immunology	37
anti-CD3-FITC (Becton Dickinson)	59
anti-CD19-RPE (Becton Dickinson)	11
anti-CD19-FITC (Becton Dickinson)	14
B. **Double Staining:**	
Bl-FITC/anti CD19-RPE	10
Leu 16-FITC/anti CD19-RPE	10
2B8 FITC/anti CD19-RPE	9
anti-CD19 FITC/anti CD19-RPE	13
Bl-FITC/anti Hu Ig RPE	10
2B8-FITC/anti Hu Ig RPE	10
B72.3-FITC/anti Hu Ig RPE	2
Leucogate Simultest	99

Immunoprecipitation of radiolabeled cellular CD20 antigen by either 2B8 or Bl resulted in the precipitation of indistinguishable doublet protein species with molecular weights of approximately 33 and 35 KD (data not shown).

2. Production and Characterization of 2B8-.MX-DTPA

The 2B8-MX-DTPA conjugate was produced by reacting the antibody with a 4:1 molar excess of isothiocyanatobenzyl-3-methyldiethylene-triaminepentaacetic acid (4). Typically, 1-2 mol of MX-DTPA chelate were introduced per mol of 2B8 antibody. As shown by the results presented in Fig. 4, the 2B8-MX-DTPA conjugate exhibited no apparent loss in immunoreactivity, *vis a vis* native 2B8, as both the native and conjugated 2B8 antibodies exhibited virtually identical Bl inhibition profiles; the IC50 values for 2B8 and 2B8-MX-DTPA were approximately 3 and 4 μg/mL, respectively. These results were obtained using ^{125}I-labeled Bl antibody in a whole-cell radioimmunoassay performed using SB cells. Similar results were obtained using 2B8 or 2B8-MX-DTPA as inhibitors of ^{125}I-labeled 2B8 binding to SB cells; both 2B8 and its MX-DTPA conjugate inhibited ^{125}I-2B8 binding to SB cells at concentrations of approximately 3-4 μg/mL (data not shown).

To assess the *in vitro* stability of the native 2B8 antibody and the 2B8-.MX-DTPA conjugate, samples in normal saline or saline containing 10 mM glycine-HCl, pH 6.8, were incubated at 4° and 30°C for 12 weeks and aliquots were assayed weekly using the following assays: immunoreactivity by whole-cell enzyme immunoassay, SDS-PAGE under reducing and non-reducing conditions, and isoelectric focusing gel electrophoresis. While immunoreactivity assays detected no loss of antigen recognition by antibody samples incubated at either temperature (Figure 5), the isoelectric focusing range for the antibody (pH 7.30-8.40 at week zero), which was stable at 4°C, did exhibit a decrease of 0.2 pH unit at 30°C after week six (Table 22). This result may be equivocal, however, as it is at the limit of experimental error for the assay.

Table 22

2B8/2B8-MX-DTPA pI SUMMARY

WEEK	2B8 4 SAL	2B8 30 SAL	2B8 4 GLY	2B8 30 GLY	2B8-MX 4 SAL	2B8-MX 30 SAL	2B8-MX 4 GLY	2B8-MX 30 GLY
0	7.46-8.37		7.46-8.37		6.30-8.21		6.30-8.21	
1	7.39-8.24	7.42-8.27	7.46-8.31	7.46-8.24	6.39-8.26	6.39-8.26	6.32-8.24	6.25-8.24
2	7.38-8.27	7.45-8.34	7.45-8.40	7.45-8.34	6.02-8.40	6.02-8.34	6.02-8.40	5.95-8.27
3	7.47-8.35	7.33-8.35	7.40-8.29	7.33-8.29	6.0-8.29	6.0-8.29	6.0-8.22	6.0-8.15
4	7.38-8.24	7.38-8.24	7.38-8.35	7.38-8.28	5.99-8.28	5.99-8.35	5.99-8.35	5.99-8.35
5	7.29-8.25	7.29-8.25	7.37-8.32	7.37-8.32	5.90-8.32	5.90-8.27	5.90-8.32	5.90-8.27
6	7.24-8.12	7.20-8.27	7.27-8.27	7.20-8.12	5.85-8.27	5.85-8.27	5.85-8.27	5.85-7.95
7	7.39-8.32	7.17-8.32	7.35-8.25	7.17-8.47	6.02-8.25		5.95-8.32	5.95-8.32
8	7.33-8.29	7.26-8.36	7.40-8.36		5.86-8.36	5.86-8.36	5.86-8.36	5.86-8.21
9	7.49-8.53	7.26-8.45	7.41-8.45	7.34-8.30	5.93-8.45	5.93-8.45	5.93-8.45	5.93-8.23
10	7.26-8.27	7.19-8.27	7.26-8.27	7.19-8.27	5.95-8.35	5.95-8.35	5.88-8.35	5.95-8.13
11	7.40-8.27	7.18-8.27	7.40-8.35	7.18-8.13	5.93-8.35	5.93-8.27	5.93-8.27	5.93-8.13
12	7.26-8.18	7.04-8.18	7.26-8.18	7.19-8.11	5.90-8.26	5.90-8.18	5.90-8.26	5.90-8.18

Samples of native 2B8 and 2B8-MX-DTPA were formulated in different buffers and incubated at either 40 or 30°C for 12 weeks. During this period various assays, including isoelectric point determinations, were performed. The values shown above show the isoelectric point range for the native and conjugated antibody incubated at each temperature, in each of the formulations, and for each of the twelve weeks during the stability study. The headings represent: 2B8 4 SAL, 2B8 incubated at 4°C in saline; 2B8 30 SAL, 2B8 incubated at 30°C in saline; 2B8 4 GLY, 2B8 incubated at 4°C in normal saline containing 10 mM glycine; 2B8 30 GLY, 2B8 incubated at 30°C in normal saline containing 10mM glycine; 2B8-MX 4 SAL; 2B8-MX-DTPA (conjugate) incubated at 4°C in saline; 2B8-MX 30 SAL, conjugate incubated at 30°C in saline; 2B8-MX 4'GLY, conjugate incubated at 4°C in normal saline containing 10 mM glycine; and, 2B8-MX 30 GLY, conjugate incubated at 30°C in normal saline containing 10 mM glycine.

Finally, using non-reducing SDS-PAGE, the 30°C antibody samples exhibited high molecular weight aggregates after week 1 (Table 23). Densitometric analyses of the gels indicated that the aggregates represented between 8 and 17% of the samples (Table 23). However, when these samples were analyzed by reducing SDS-PAGE, no evidence of the high molecular weight species was found, suggesting the formation of covalent antibody aggregates at 30°C. Again, no loss of immunoreactivity was observed.

Table 23

In Vitro Stability of 2B8

A. Desensitometric Scans of Non-Reducing SDS Gels

Sample	Percentage		
	High MW	Monomer	Low MW
Reference	0	100.00	0
12 wk/4°C/saline	0	95.42	4.58
12 wk/4°C/glycine	0	100.00	0
12 wk/30°C/saline	7.63	83.34	9.03
12 wk/30°C/glycine	16.70	72.11	11.18

B. Desensitometric Scans of Reducing SDS Gels

Sample	Percentage		
	High MW	Monomer	Low MW
Reference	0	100.00	0
12 wk/30°C/saline	0	100.00	0
12 wk/30°C/glycine	0	10.00	0

During the course of this stability study, samples of 2B8-MX-DTPA, incubated at both 4° and 30°C were also tested for radiometal incorporation using ^{90}Y. Samples assayed at weeks 4, 8, and 12 incorporated >90% of the ^{90}Y, regardless of the incubation temperature.

Finally, in a separate study, aliquots of 2B8-MX-DTPA incubated at 4° and 30° C for 10 weeks were radiolabeled with ^{111}In and their tissue biodistribution assessed in BALB/c mice. Conjugate from both incubation temperatures produced similar biodistributions (data not shown). Moreover, the results obtained were similar to biodistribution results obtained in BALB/c mice using ^{111}I-labeled conjugate stored at 4°C (see below).

The radiolabeling protocols for both ^{111}In and ^{90}Y were found to be reproducible. Typically, radioincorporations of >95% for ^{111}In and >90% for ^{90}Y were obtained. Specific activities for ^{111}I- and ^{90}Y-labeled conjugates were routinely in the range of 2-3 and 10-15 mCi/mg antibody, respectively. In initial development of the ^{111}I- and ^{90}Y radiolabeling protocols, uncomplexed radioisotopes were removed from the radiolabeled 2B8-MX-DTPA using HPLC gel permeation chromatography. In later experiments, HPLC purification of the indium-labeled conjugate was eliminated because of the high radioincorporations obtained (>95%) with this isotope.

The immunoreactivity of ^{111}In and ^{90}Y-labeled preparations of 2B8-MX-DTPA were analyzed by the method of Lindmo (3). The ^{111}In labeled 2B8-MX-DTPA was found to be 100% immunoreactive (Fig. 6), and the ^{90}Y-labeled conjugate was determined to be 60% immunoreactive (data not shown).

3. Characterization of ^{111}I- and ^{90}Y-Labeled 2B8-MX-DTPA

Preliminary experiments with the ^{90}Y-labeled conjugate demonstrated that significant antibody degradation and loss of immunoreactivity occurred at specific activities >10 mCi/mg antibody. Therefore, a formulation was developed to

minimize the effects of radiolysis. While a number of low molecular weight free-radical scavengers were evaluated and found to be effective, high concentrations of human serum albumin (HSA) were the most effective in preserving antibody integrity and immunoreactivity (Figures 7-9).

The ^{90}Y-labeled antibody was formulated in 1X PBS, pH 7.4 containing 75 mg/mL HSA; diethylenetriaminepentaacetic acid (DTPA) was also added to a final concentration of 1 mM to insure that any ^{90}Y which may be lost from the antibody would be chelated. Degradation of 2B8-MX-DTPA, radiolabeled to a specific activity of 14.6 mCi/mg was evaluated at 0 and 48 hours using SDS-PAGE and autoradiography. Figures 8 and 9 show that the radiolabeled antibody exhibited no significant degradation over a period of 48 h when incubated at 4°C. Analysis using instant thin layer chromatography showed that the loss of ^{90}Y was less than 2% during the 48 h incubation (Table 24). The immunoreactivity was also relatively constant at 60% (Table 24).

Table 24

Stability of Clinically-Formulated ^{90}Y-2B8-MX-DTPA

Time (Hours at 4°C)	Percent Conjugate-Associated Radioactivity	Percent Immunoreactivity
0	97.2	62
24	96.2	60
48	96.2	60

Radiolabeled conjugate (14.6 mCi/mg specific activity) was formulated in PBS, pH 7.4, containing 75 mg/mL human serum albumin and 1 MM DTPA and aliquots incubated at 4°C. Conjugate stability was analyzed at the times shown by SDS-PAGE and autoradiography, instant thin-layer chromatography and by whole-cell binding assay. The results show that approximately 96% of the radiometal remained associated with the conjugate after 48 hours at 4°C, and that antibody immunoreactivity remained constant at approximately 60%.

Formulation studies were also performed with the ^{111}In-labeled conjugate; the specific activity was 2.2 mCi/mg. The radiolabeled antibody was evaluated in 1X PBS, pH 7.4 containing 50 mg/mL HSA. Figure 10 shows photographs of the autoradiograms for time zero and 48 h incubation samples; densitometric analysis of the autoradiograms indicate that there was no degradation of the radiolabeled antibody over the course of the study (Figures 11, 12). Instant thin-layer chromatography analysis of the samples demonstrated no loss of ^{111}In (Table 25); moreover, immunoreactivity was maintained at approximately 100% (Table 25).

Table 25

Stability of Clinically-Formulated ^{111}In-2B8-MX-DTPA

Time (Hours at 4°C)	Percent Conjugate-Associated Radioactivity	Percent Immunoreactivity
0	94.0	105
24	96.5	104
48	96.0	100

The radiolabeled conjugate (2.2 mCi/mg specific activity) was formulated in PBS, pH 7.4, containing 50 mg/mL human serum albumin and aliquots incubated at 4°C. Conjugate stability was analyzed by SDS-PAGE and autoradiography, by instant thin-layer chromatography, and by whole-cell binding assay. The results show that approximately 96% of the radiolabel was retained with the conjugate after 48 hours at 4°C, and that antibody immunoreactivity remained constant at approximately 100%.

When a clinically-formulated preparation of 2B8-MX-DTPA, radiolabeled with ^{90}Y to a specific activity 14.6 mCi/mg, was incubated for 96 hours at 37°C in human serum and analyzed by non-reducing SDS-PAGE and autoradiography, less than 4% of the radioisotope was lost during the course of the incubation period. Densitometric scans of the autoradiograms at time zero and 96 h indicated no significant degradation of the radiolabeled conjugate (Figures 13-15). These results were corroborated by analytical thin-layer chromatographic analyses of the time zero and 96-hour samples (Table 26). Taken together these results suggest that the yttrium-labeled conjugate is stable under the conditions used in this study. Similar results were obtained with the ^{111}In labeled 2B8-MX-DTPA conjugate (Figures 16-18).

Table 26

Analytical Thin-Layer Chromatographic Analysis of ^{90}Y-2B8-MX-DTPA Conjugate Incubated in Human Serum for 96 Hours at 37°C

Time (Hours at 37°C)	Percent Conjugate-Associated Radioactivity
0	95.1
24	95.2
48	93.2
72	92.0
96	91.4

Human serum samples containing ^{90}Y-2B8-MX-DTPA (specific activity 14.6 mCi/mg) were analyzed at the times shown by spotting 1 μl of a 1:20 dilution of samples on instant thin-layer chromatography strips; samples were analyzed in triplicate. Chromatography strips were developed by ascending chromatography in 10% ammonium acetate in methanol:water (1:1; v/v), dried, and cut in half crosswise. The radioactivity associated with the bottom and top halves of each strip was then determined and the percent conjugate-associated radioactivity calculated. (Free radiometal migrates with the solvent front while protein-associated radioactivity remains at the origin.) The means of each determination of conjugate-associated radioactivity are shown.

B. Animal Studies.

1. High-Dose Pharmacology/Toxicology Studies with 2B8 and 2B8-MX-DTPA

In a GLP study performed at White Sands Research Center (Study Number 920111), cynomolgus monkeys were given intravenous injections of various doses of 2B8. Blood samples were taken before each new injection and the blood was processed for flow cytometric evaluation of the lymphocyte populations (Table 27).

Table 27

Primate B cell Populations Determined by Flow Cytometry, Following Infusion of Anti-CD20 Murine Monoclonal Antibody 2B8

	Animal #	Dose	Day	B cells[a,b]	% Depletion
Group I					
	452	saline	0	20.1	0
			1	18.3	9
			7	21.6	0
			13	14.6	27
			38	15.5	23
			52	18.6	7
	424	saline	0	12.4	0
			1	11.6	6
			7	11.2	10
			13	8.4	32
			38	7.7	38
			52	13.1	0
Group II					
	540	0.6 mg/kg	0	16.1	0
			1	7.1	54
			7	6.0	63
			13	5.7	65
			38	10.8	33
			52	14.4	11
	804	0.6 mg/kg	0	17.6	0
			1	8.3	53

	Animal #	Dose	Day	B cells[a,b]	% Depletion
			7	6.1	65
			13	6.6	62
			38	5.1	71
			52	5.2	68
Group III					
	701	2.5 mg/kg	0	21.6	0
			1	10.7	50
			7	3.0	86
			13	10.7	50
	754	2.5 mg/kg	0	19.9	0
			1	11.2	44
			7	10.5	47
			13	9.0	55
Group IV					
	782	10 mg/kg	0	15.9	0
			1	3.0	81
			7	3.5	78
			13	6.5	59
	164	10 mg/kg	0	17.7	0
			1	8.4	47
			7	7.9	50
			13	7.7	42
Group V					
	705	10 mg/kg	0	17.2	0
			1	5.2	70
			7	1.3	69

Animal #	Dose	Day	B cells[a,b]	% Depletion
		13	8.2	52
		38	17.1	1
		52	13.3	22
716	10 mg/kg	0	34.7	0
		1	18.6	46
		7	8.1	77
		13	3.5	90
		38	6.9	80
		52	9.2	61

[a]Percent of total lymphocytes.

[b]B cell population quantitated by double staining marker reagents anti mouse IGG-RPE + anti human IG-FITC (anti mouse IgG RPE detects 2B8 blocked CD20 and anti human IgG FITC detects monkey B cell surface Ig)

Animals in groups I through IV were injected every 48 hours for a total of seven injections; animals in group V were injected once on day 0. The animals in Groups III and IV were sacrificed on day 14.

No significant pharmacotoxic effects related to the administration of the anti-CD20 antibody 2B8 were noted in any clinical parameter evaluated during or following the study. Similarly, no abnormalities were noted during analysis of the various histopathology specimens obtained from animals in groups III and IV.

The study duration was 14 days and the animals were evaluated during the study in the following categories: clinical observations, body weights, body temperature, food and water intake, fecal elimination, serum chemistries, hematology, urinalysis, and physical examinations. Additionally, the animals in each group were bled on days 0, 1, 7, and 13 and the blood analyzed for serum antibody (2B8) levels and for T- and B-cell levels. On day 13 the animals in Groups III and IV were sacrificed and selected tissues examined by light microscopy following specimen preparation. The tissues evaluated were: heart, spleen, liver, kidney, lung, cerebral cortex, spinal cord, lymph node, stomach, ileum, colon, skeletal muscle, testis/ovary, pancreas, and bone marrow.

When the blood from the treated animals was analyzed for levels of circulating T- and B-cells, animals in groups II through V exhibited >50% loss of circulating B-cells through day 13 (Fig. 19); administration of the antibody had no effect on T-cell levels (data not shown). All groups receiving 2B8 showed saturation of B-cells and excess antibody in the plasma (not shown). The animals in group V, which received a single 10.0 mg/Kg dose of 2B8 also exhibited reduction in circulating B-cell levels equivalent to that observed in animals in the other groups.

The animals in groups I, II, and V were examined through day 52 (Fig. 20). The levels of B-cells returned to > 70% of normal by day 38, except for one animal in Group II (PRO804) and one animal in Group V (PRO716). The levels of circulating B-cells in these animals remained at approximately 40% of normal levels after 52 days.

In addition to this study, the pharmacotoxic effects of ^{89}Y-2B8-MX-DTPA were assessed in cynomolgus monkeys in a GLP study performed at White Sands Research Center (Study No. 920611). Clinical-grade conjugate was loaded with non-radioactive ^{89}Y. The yttrium-bearing conjugate was formulated in PBS pH 6.8, containing 75 mg/mL human serum albumin and 1mM DTPA (clinical formulation) and administered intravenously as described in the Methods Section.

As shown by the results in Figure 21, the ^{89}Y-labeled 2B8-MX-DTPA had little, if any, effect on circulating B-cells in these animals, regardless of the dose administered. In addition, other than a general depletion of lymphocytes (20-43%), no significant abnormalities were found in any clinical parameter evaluated, including serum chemistry, urinalysis, body weights and temperatures.

2. Pharmacokinetic Studies with 2B8 and 2B8-MX-DTPA

As described above, the animals in group V of the GLP study received a single dose of 10.0 mg/Kg of 2B8. Linear regression analysis of the data suggest that the native antibody was cleared from the circulation of these monkeys with a 13 tl/2 value of approximately 4.5 days. In a similar study using BALB/c mice, the β $t_{1/2}$ values for native and conjugated 2B8 were determined by linear regression analysis (not shown) to be 8.75 days (Fig. 22). These results suggest that conjugation of 2B8 had no effect on its clearance from BALB/c mice.

3. Biodistribution and Tumor Localization Studies with Radiolabeled 2B8-MX-DTPA

Building on the preliminary biodistribution experiment described above (Section 2d), conjugated 2B8 was radiolabeled with ^{111}In to a specific activity of 2.3 mCi/mg and roughly 1.1 μCi was injected into each of twenty BALB/c mice to determine biodistribution of the radiolabeled material. Subsequently, groups of five mice each were sacrificed at 1, 24, 48 and 72 hours and their organs and a

portion of the skin, muscle and bone were removed and processed for analysis. In addition, the urine and feces were collected and analyzed for the 24-72 hour time-points. The level of radioactivity in the blood dropped from 40.3% of the injected dose per gram at 1 hour to 18.9% at 72 hours (Tables 1-4; Fig. 23). Values for the heart, kidney, muscle and spleen remained in the range of 0.7-9.8% throughout the experiment. Levels of radioactivity found in the lungs decreased from 14.2% at 1 hour to 7.6% at 72 hours; similarly the respective liver injected dose per gram values were 10.3% and 9.9%. These data were used in determining radiation absorbed dose estimates ^{111}In-2B8-MX-DTPA (Table 19).

The biodistribution of ^{90}Y-labeled conjugate, having a specific activity of 12.2 mCi/mg antibody, was evaluated in BALB/c mice. Radioincorporations of >90% were obtained and the radiolabeled antibody was purified by HPLC. Tissue deposition of radioactivity was evaluated in the major organs, and the skin, muscle, bone, and urine and feces over 72 hours and expressed as percent injected dose/g tissue. The results shown in Tables 5-8 and Figure 24 demonstrate that while the levels of radioactivity associated with the blood dropped from approximately 39.2% injected dose per gram at 1 hour to roughly 15.4% after 72 hours; the levels of radioactivity associated with tail, heart, kidney, muscle and spleen remained fairly constant at 10.2% or less throughout the course of the experiment. Importantly, the radioactivity associated with the bone ranged from 4.4% of the injected dose per gram bone at 1 hour to 3.2% at 72 hours. Taken together, these results suggest that little free yttrium was associated with the conjugate and that little free radiometal was released during the course of the study. These data were used in determining radiation absorbed dose estimates for ^{90}Y-2B8-MX-DTPA (Table 20).

For tumor localization studies, 2B8-MX-DTPA was prepared and radiolabeled with ^{111}In to a specific activity of 2.7 mCi/mg. One hundred microliters of labeled conjugate (approximately 24 μCi) were subsequently injected into each of 12 athymic mice bearing Ramos B-cell tumors. Tumors ranged in weight from 0.1 to 1.0 grams. At time points of 0, 24, 48, and 72 hours following injection, 50 μL of blood was removed by retro-orbital puncture, the mice sacrificed by cervical dislocation, and the tail, heart, lungs, liver, kidney, spleen, muscle, femur, and tumor removed. After processing and weighing the tissues, the radioactivity associated with each tissue specimen was determined using a gamma counter and the values expressed as percent injected dose per gram.

The results (Fig. 25) demonstrate that the tumor concentrations of the ^{111}In-2B8-MX-DTPA increased steadily throughout the course of the experiment. Thirteen percent of the injected dose was accumulated in the tumor after 72 hours. The blood levels, by contrast, dropped during the experiment from over 30% at time zero to 13% at 72 hours. All other tissues (except muscle) contained between 1.3 and 6.0% of the injected dose per gram tissue by the end of the experiment; muscle tissue contained approximately 13% of the injected dose per gram.

C. Dosimetry

The summary dosimetry data derived from biodistribution studies in normal BALB/c mice and presented in Tables 19 and 20, for the indium- and yttrium-labeled conjugates, respectively, are in agreement with data presented in the literature when compared per millicurie of injected dose (5) and suggest that both the yttrium- and indium-labeled conjugates of 2B8 may be safely evaluated for clinical efficacy in lymphoma patients.

D. Toxicology.

1. 2B8: Single Dose General Safety Test.

Mice and guinea pigs were administered a single intra peritoneal dose of 2B8 (0.5 mL or 5.0 mL, respectively) and observed for seven days. No overt signs of toxicity were detected.

2. 2B8 and 2B8-MX-DTPA: Immunohistology Studies with Human Tissues.

The tissue reactivity of murine monoclonal antibody 2B8 was evaluated using a panel of 32 different human tissues fixed with acetone. Antibody 2B8 reacts with the anti-CD20 antigen which had a very restricted pattern of tissue distribution, being observed only in a subset of cells in lymphoid tissues including those of hematopoietic origin.

In the lymph node, immunoreactivity was observed in a population of mature cortical B-lymphocytes as well as proliferating cells in the germinal centers. Positive reactivity was also observed in the peripheral blood, B-cell areas of the tonsils, white pulp of the spleen, and with 40-70% of the medullary lymphocytes found in the thymus. Positive reactivity was also seen in the follicles of the lamina propria (Peyer's Patches) of the large intestines. Finally, aggregates or scattered lymphoid cells in the stroma of various organs, including the bladder, breast, cervix, esophagus, lung, parotid, prostate, small intestine, and stomach, were also positive with antibody 2B8.

All simple epithelial cells, as well as the stratified epithelia and squamous epithelia of different organs, were found to be unreactive. Similarly, no reactivity was seen with neuroectodermal cells, including those in the brain, spinal cord and peripheral nerves. Mesenchymal elements, such as skeletal and smooth muscle cells, fibroblasts, endothelial cells, and polymorphonuclear inflammatory cells were also found to be negative.

The tissue reactivity of the 2B8-MX-DTPA conjugate was evaluated using a panel of sixteen human tissues which had been fixed with acetone. As previously demonstrated with the native antibody, the 2B8-MX-DTPA conjugate recognized the CD20 antigen which exhibited a highly restricted pattern of distribution, being found only on a subset of cells of lymphoid origin. In the lymph node, immunoreactivity was observed in the B-cell population. Strong reactivity was seen in the white pulp of the spleen and in the medullary lymphocytes of the thymus. Immunoreactivity was also observed in scattered lymphocytes in the bladder, heart, large intestines, liver, lung, and uterus, and was attributed to the presence of inflammatory cells present in these tissues. As described with the native antibody (above), no reactivity was observed with neuroectodermal cells or with mesenchymal elements.

III. Discussion

The murine monoclonal anti-CD20 antibody 2B8, produced by a clone with the same designation, exhibits an affinity for the B-cell CD20 antigen which may be higher than that observed for the B1 antibody, as determined by competition with antibodies of known specificity for the CD20 antigen, and by Scatchard analysis. Further, immunoprecipitation data suggest that the antigen precipitated by 2B8 appears to be the same antigen as the one precipitated by B1, as both antibodies precipitated a doublet with relative molecular weights of 33 and 35 KD. Cytofluorographic analysis of the specificity of the 2B8 antibody for peripheral blood lymphocytes demonstrates that the antibody reacts specifically with B-cells and has no demonstrated reactivity with T-cells or other types of lymphocytes. Finally, preliminary stability data suggest that the antibody is stable at 30°C for 12 weeks with no loss of immunoreactivity.

When the 2B8 antibody was conjugated to methylbenzyl diethylenetriamine-pentaacetic acid (MX-DTPA), virtually no reduction in immunoreactivity, relative

to the native antibody, was observed. Further, radiolabeling the conjugate with either ^{111}In or ^{90}Y produced labeled conjugates with immunoreactivities of 100% and 60%, respectively. Stability studies of ^{111}In or ^{90}Y-labeled conjugates incubated in human serum for 96 hours at 37°C indicated negligible loss of the radiometal during the course of the study, suggesting that the conjugates will be stable when used clinically.

Tumor localization studies in athymic mice using an indium-labeled preparation of 2B8-MX-DTPA demonstrated that increasing amounts of the conjugate bound to the tumor cells during the course of the experiment without unusual accumulations in other tissues. Moreover, dosimetry estimates derived from the biodistribution. Moreover, dosimetry estimates derived from the biodistribution studies are in agreement with data published in the literature. Finally, human tissue cross-reactivity studies with the native and conjugated antibodies indicated that both antibodies recognize an antigen with highly restricted tissue distribution, reacting only with a subset of cells in lymphoid tissues, including those of hematopoietic origin. Taken together, these results suggest that conjugation did not alter the tissue specificity of the antibody, and that the radiolabeled conjugates are stable in vivo and recognize the CD20 antigen present on the surface of tumors produced experimentally in athymic mice.

When 2B8 was used in a high-dose pharmacology/toxicology study, the antibody produced no significant pharmacotoxic effects in any parameter evaluated, either during or following the study. Similarly, no abnormalities were noted during analysis of the various histopathology specimens examined by light microscopy. Surprisingly, all doses of the antibody used produced marked depletion of circulating B-cells. Circulating B-cell levels did, however, return to roughly normal levels once administration of the antibody ceased. In the single-dose group of monkeys (Group V) the native antibody was cleared from the circulation with an apparent β $t_{1/2}$ value of approximately 4.5 days. Predictably,

when this pharmacokinetic study was performed in BALB/c mice the 2B8 antibody was cleared with a $\beta\ t_{1/2}$ value of 8.75 days. Thus, taken together, these data suggest that the native antibody may also provide some clinical effect when administered as an adjunct to the radiolabeled conjugates.

Overall our data indicate that the high affinity 2B8 antibody and its MX-DTPA conjugate exhibit a restricted pattern of human tissue reactivity. Moreover, in primates, the native antibody is non-toxic and produces transient clearance of B-cells; however, once the antibody is cleared from the circulation the B-cell levels return reasonably rapidly. Additionally, the indium- and yttrium-labeled 2B8-MX-DTPA conjugates appeared stable *in vitro*, exhibiting no loss of radiometal during prolonged incubation in human serum. Finally, radiation dose estimates derived from the biodistribution of ^{90}Y- or ^{111}In-labeled 2B8-MX-DTPA in BALB/c mice are in agreement, per millicurie of injected dose, with dose estimates derived from human clinical studies using conjugated anti-shared idiotype antibodies radiolabeled with these isotopes.

IV. SUMMARY OF PRE-CLINICAL DEVELOPMENT OF "MIX-&-SHOOT" RADIOLABELING PROTOCOL FOR PREPARATION OF ^{90}Y-2B8-MX-DTPA

A. Introduction

A ^{90}Y-labeled murine monoclonal anti-CD20 antibody (2B8) has been evaluated in a Phase I clinical trial for the treatment of relapsed B-cell lymphoma. The original protocol used for the preparation of the yttrium-labeled antibody used a high performance liquid chromatographic (HPLC) step for removal of non-protein bound radioisotope prior to formulation and administration to patients. Unfortunately, this process is particularly time consuming, resulting in a longer exposure of the antibody to radioisotope in an unprotected state. This results in

increased radiolysis of the antibody with a concomitant decrease in immunoreactivity. Additionally, the laborious aspect of the process makes it difficult to prepare more than one dose per day at the radiopharmacy. Simplification of the process would expedite implementation at the clinical site as an alternative to using NIPI Pharmacy Services as a radiopharmacy.

Accordingly, a revised radiolabeling procedure was developed, referred to as the "mix-and-shoot" method, which obviates the need for HPLC purification while maintaining a high radioincorporation and improved retention of immunoreactivity. *In vitro* stability studies as well as biodistribution studies in mice showed that radiolabeled antibody prepared using the "mix-and-shoot" method is comparable to material produced using the current HPLC process. The results of these pre-clinical studies indicate that this new "mix-&-shoot" protocol can be used to prepare ^{90}Y-labeled 2B8-MX-DTPA suitable for use in clinical trials.

B. Materials and Methods

Materials

1. Cells

The human lymphoblastic cell lines SB (CD20 positive) and HSB (CD20 negative;) were obtained from the American Type Culture Collection and maintained in RPMI-1640 containing 10% fetal bovine serum and supplemented with glutamine.

2. Antibodies

The 2B8 antibody was purified by the Manufacturing department from hollow-fiber bioreactor supernatant using protocols previously described in the IND (BB-IND 4850/4851).

3. Additional Reagents

Yttrium-[90] chloride was obtained from Amersham. All other reagents were obtained from sources described in the appended reports cited below. Reagents used for radiolabeling protocols were processed to remove contaminating heavy metal ions which could compete with-the radioisotopes during the radiolabeling step (see Methods section). Reagents were made under GMP conditions by IDEC's- Manufacturing department following established Batch Production Records.

Methods

1. Preparation of 2B8-D4X-DTPA

Clinical-grade MX-DTPA was obtained from Coulter Immunology as the disodium salt in water and stored at -70°C. Conjugate (2B8-MX-DTPA) was prepared by the Manufacturing department. Two different lots of conjugate were used in these studies; both were provided in normal saline at 10 mg/mL. The conjugates were filled in sterile 2 mL polypropylene syringes and stored at 2-8°C.

2. Maintenance of Metal-Free Conditions

All manipulations of reagents were performed to minimize the possibility of metal contamination. Polypropylene or polystyrene plastic containers such as flasks, beakers and graduated cylinders were used. These were washed with Alconox and exhaustively rinsed with Milli-Q water or Water for Irrigation (WFIr) before use. Metal-free pipette tips (BioRad) were used for accurately manipulating small volumes. Larger volumes of reagents were manipulated using sterile, plastic serological pipettes. Reactions were conveniently performed in 1.8 mL screw-top microfuge tubes made from polypropylene.

3. Determination of Radioincorporation

Radioincorporation was determined using instant thin-layer chromatography (ITLC) in triplicate according to SOP SP-13-008. In general, the protocol was as follows: radiolabeled conjugate was diluted 1:20 in 1X PBS containing 1 mM

DTPA or 5 mM EDTA, then 1 μ-L spotted 1.5 cm from one end of a 1 x 5 cm strip of ITLC SG paper (Gelman Sciences). The paper was developed using 10% ammonium acetate in methanol:water (1:1;v/v). The strips were dried, cut in half crosswise, and the radioactivity associated with each section determined by scintillation counting. The radioactivity associated with the bottom half of the strip (protein-associated radioactivity) was expressed as a percentage of the total radioactivity determined by summing the values for both top and bottom halves.

4. "Mix and-Shoot" Protocol for Yttrium-[90]-Labeled 2B8-MX-DTPA

Antibodies were radiolabeled with carrier-free ^{90}Y chloride provided by Amersham in 0.04 M HCl. An aliquot of radioisotope (10-20 mCi/mg antibody) was transferred to a polypropylene tube and 0.02X volume of metalfree 2 M sodium acetate was added to adjust the solution to pH 3.6. 2B8-NaDTPA (0.3 mg; 10.0 mg/mL in normal saline) was added immediately and the solution gently mixed. The solution was checked with pH paper to verify a pH of 3.8-4.1 and incubated for 5 min. The reaction was quenched by transferring the reaction mixture to a separate polypropylene tube containing 1XPBS with 75 mg/mL human serum albumin (HSA) and 1 mM diethylenetriaminepentaacetic acid (DTPA) and gently mixed. The radiolabeled antibody was stored at 2-8°C.

Specific activities were determined by measuring the radioactivity of an appropriate aliquot of the radiolabeled conjugate. This value was corrected for the counter efficiency, related to the protein concentration of the conjugate, determined by absorbance at 280 nm and expressed as mCi/mg proteins.

5. *In Vitro* Immunoreactivity of Yttrium-[90]-2B8-MX-DTPA

Immunoreactivity of ^{90}Y-labeled conjugate was determined using SOP #SP13-009 based on a modified version of the whole-cell binding assay described by Lindmo. Increasing concentrations of log phase, CD20-positive SB cells or CD20-negative HSB cells were added to duplicate sets of 1.5 mL polypropylene

tubes; final volume of cells, 0.40 mL. The radiolabeled conjugate was diluted to a final antibody concentration of 1-2.5 ng/mL and 0.35 mL was added to each tube. Following a 90 min incubation, the cells were pelleted by centrifugation and the supernatants collected. Radioactivity remaining in the supernatant fraction was determined with a scintillation counter. Data were plotted as the quotient of the total radioactivity added divided by the cell-associated radioactivity versus the inverse of the cell number per tube. The y axis intercept represents the immunoreactive fraction.

6. *In Vitro* Stability of Clinically-Formulated Yttrium-[90]-2B8-MX-DTPA

The 2B8-MX-DTPA conjugate was radiolabeled with ^{90}Y and formulated as described in the "mix & shoot" protocol provided above. Two lots of radiolabeled conjugate were prepared; one lot was used for assessing radioincorporation stability and the other lot used to assess retention of immunoreactivity. The formulated conjugates were incubated at 4°C for 48 hours and aliquots analyzed at time 0, 24 h and 48 hours using non-reducing SDS-PAGE and autoradiography. Immunoreactivity at each time point was assessed using the assay described above.

7. *In Vitro* Stability of Yttrium-[90]-2B8-NTX-DTPA in Human Serum

The stability of ^{90}Y-labeled 2B8-MX-DTPA was assessed by incubation in human serum at 37°C for up to 72 hours. The conjugated antibody was radiolabeled with yttrium-[90] and formulated as described above. The radiolabeled conjugate was diluted 1:10 with normal human serum (non-heatinactivated) and aliquots incubated in plastic tubes at 37°C. At selected times, samples were removed and analyzed by non-reducing SDS-PAGE and autoradiography.

8. Biodistribution of Yttrium-[90]-2B8-MX-DTPA

Yttrium-[90]-labeled 2B8-MX-DTPA was evaluated for tissue biodistribution in eight to ten week old BALB/c mice. The radiolabeled conjugate

was prepared and formulated as described above. Mice were injected intravenously with 5 μCi of ^{90}Y-labeled 2B8-MX-DTPA and groups of five mice were sacrificed at 1, 24, 48, and 72 hours. After sacrifice, the tail, heart, lungs, liver, kidney, spleen, muscle, femur were removed, washed, weighed; a sample of blood and skin were also removed for analysis. Radioactivity associated with each tissue sample was determined by measuring bremstrahlung radiation using a gamma counter and the percent injected dose per gram tissue and percent injected dose per organ determined.

9. Dosimetry

Biodistribution data obtained using mice injected with ^{90}Y-labeled 2B8-MX-DTPA were used to calculate estimates of the radiation doses absorbed from a 1.0 mCi dose administered to a 70 Kg patient. Estimates were made according to methods adopted by the Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine. These calculations were performed Mr. Phillip Hagan, Nuclear Medicine Service, VA Medical Center, La Jolla, CA 92161.

10. Validation of Protocol for Preparation of Clinical Doses of Yttrium-[90]-2B8-MX-DTPA

(Reference R&D report titled "Validation of "Mix-and-Shoot" Radiolabeling Protocol for the Preparation of Clinical Doses of ^{90}Y-2B8-MX-DTPA; author, P. Chinn; dated April 22, 1994).

C. Results

1. Preparation of Yttrium-[90]-Labeled 2B8-MX-DTPA Using "Mix-&-Shoot" Protocol

Preliminary experiments evaluating the kinetics of the radiolabeling reaction with 2B8-MX-DTPA and ^{90}Y showed that at pH 3.6-4.0, 95% of the radioisotope was incorporated for a reaction time of 5 to 10 min. The reproducibility of this radioincorporation (95.7% $\pm$ 1.7%) was subsequently confirmed in a validation

study for the scale-up protocol (Reference R&D report titled "Validation of "Mix-and-Shoot" Radiolabeling Protocol for the Preparation of Clinical Doses of ^{90}Y-2B8-MX-DTPA; author, P. Chinn; dated April 22, 1994). The preparation of ^{90}Y-labeled 2B8-MX-DTPA using this "mix-&-shoot" protocol gave a product comparable to that produced with the, HPLC method (see BB-IND 4850/4851). The radiolabeling protocol was found to be reproducible with specific activities typically ranging from 10 to 15 mCi/mg antibody.

The immunoreactivity of the ^{90}Y-labeled 2B8-MX-DTPA prepared using this protocol was typically greater than 70%, compared with the 55-60% observed for the validations runs for the HPLC protocol (Figure 26). This difference is probably due to the reduced effects of radiolysis because of the reduced incubation time with the "mix-and-shoot" protocol. This result was typical, and, as discussed below, was representative of the validation runs for the scale-up protocol for preparing clinical doses of the radiolabeled conjugate.

2. *In vitro* Stability of ^{90}Y-Labeled 2B8-MX-DTPA

Preliminary experiments with unprotected ^{90}Y-labeled antibody conjugate prepared using the HPLC process demonstrated that radiolysis caused significant antibody degradation and loss of immunoreactivity. Therefore, a formulation buffer was developed to minimize the effects of radiolysis. Human serum albumin (HSA) was shown to be effective in minimizing. antibody degradation due to radiolysis. An evaluation was made with the radiolabeled conjugate prepared with the "mix-&-shoot" method to confirm the efficacy of the formulation in minimizing radiolysis. The ^{90}Y-labeled antibody, radiolabeled to a specific activity of 14.5 mCi/mg antibody, was formulated in 1X PBS, pH 7.4 containing 75 mg/mL HSA and 1 mM DTPA. Degradation of the conjugate 2B8-MX-DTPA was evaluated at 0, 24, and 48 hours using SDS-PAGE and autoradiography. Figures 2, 3, and 4 show that the radiolabeled conjugate exhibited no significant degradation over a period of 4S h when incubated at 4°C. Instant thin-layer chromatography analysis

showed no loss of ^{90}Y during the 48 h incubation; these results were corroborated by SDS-PAGE/autoradiographic analysis (Table 28). The immunoreactivity also was relatively constant at >88% (Table 29).

Table 28

Stability of "Mix-&-Shoot" ^{90}Y-2B8-MX-DTPA in PBS Containing Human Serum Albumin and DTPA

	Percent of Conjugate-Associated Radioactivity	
Time (h)	ITLC	SDS/PAGE
0	92.9	96.0
24	95.5	95.4
48	91.3	94.6

Table 29

Immunoreactivity of "Mix-&-Shoot" ^{90}Y-2B8-MX-DTPA in PBS Containing Human Serum Albumin and DTPA

Time (Hours at 4°C)	Percent Immunoreactivity
0	87.9
24	88.5
48	90.4

A clinically-formulated ^{90}Y-labeled 2B8-MX-DTPA at a specific activity 15.7 mCi/mg was incubated for 72 hours at 37°C in human serum. Samples analyzed by non-reducing SDS-PAGE and autoradiography (Figure 30) showed no loss of radioisotope during the course of the incubation period (Table 30). Densitometric scans of the autoradiograms at time zero and 72 h indicated no significant degradation of the radiolabeled conjugate (Figures 31 and 32). These

results were corroborated by thin-layer chromatographic analyses (Table 30). It should be noted that the radioincorporation for the antibody used in this study was lower than that obtained in the validation studies of the labeling protocol. This lower radioincorporation was due to the reduced quality of the batch of ^{90}Y chloride used for this particular preparation of radiolabeled antibody. The lower radioincorporation did not alter the conclusion that the yttrium-labeled conjugate prepared with the "mix-and-shoot" method is stable under these incubation conditions.

Table 30

Stability of ^{90}Y-2B8-MX-DTPA Conjugate Incubated in Human Serum

Time (Hours at 37°C)	Percent Conjugate-Associated Radioactivity	
	ITLC	SDS-PAGE/Autoradiography
0	85.7	88.8
24	76.4	90.0
72	87.6	88.7

Human serum samples containing ^{90}Y-2B8-MX-DTPA (specific activity 15.7 mCi/mg) were analyzed for non-protein bound ^{90}Y at the times shown using instant thin-layer chromatography strips and SDS-PAGE/autoradiography.

3. Biodistribution Studies with Yttrium-[90] 2B8-MX-DTPA

The biodistribution of the ^{90}Y-labeled conjugate, with a specific activity of 11.5 mCi/mg antibody and a radioincorporation of >95%, was evaluated in BALB/c mice. Deposition of radioactivity in tissues was evaluated for major organs, skin, muscle, bone, urine and feces over 72 hours and expressed as percent injected dose per g tissue and as percent injected dose per organ. The results

shown in Tables 31-34 and Figure 33 show that the levels of radioactivity associated with the blood decreased from approximately 43% injected dose per gram (%ID/g) at 1 hour to approximately 16% after 72 hours; at 24 h and later, the levels of radioactivity associated with heart, kidney, and spleen remained fairly constant at 4-8%. For lung and liver, radioactivity decreased from 10-12% at 1 h to 8%-10% at 72 h. For the skin, radioactivity was relatively constant at approximately 3% from 24 h through 72 h. The radioactivity in the gastrointestinal tract was constant at 0.5-1% from 24 h to 72 h. Radioactivity for muscle remained approximately 0.6% throughout the course of the study. The uptake of radioactivity by femur (bone) remained less than 4% at all time points indicating that the amount free yttrium in the conjugate preparation was negligible and that little free radiometal was released during the course of the study.

Table 31

Distribution of Activity 1.0 Hour Following I.V. Injection of ^{90}Y-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.37 ± 0.053	42.74 ± 0.78	58.52 ± 1.74
Heart	0.101 ± 0.01	8.03 ± 3.33	0.82 ± 0.37
Lung (2)	0.126 ± 0.01	12.44 ± 0.94	1.56 ± 0.05
Kidney (1)	0.129 ± 0.01	7.81 ± 1.24	0.997 ± 0.10
Liver	0.899 ± 0.07	10.08 ± 1.28	9.01 ± 0.52
Spleen	0.077 ± 0.004	10.74 ± 0.96	0.823 ± 0.04
Muscle	7.83 ± 0.28	0.44 ± 0.08	3.43 ± 0.51
Bone	2.94 ± 0.11	3.44 ± 0.57	10.11 ± 1.80
Skin	2.94 ± 0.11	1.46 ± 0.58	4.24 ± 1.57
GI Tract	2.33 ± 0.08	1.02 ± 0.19	2.36 ± 0.35
Urine	--	--	--
Feces	--	--	--
		TOTAL	94.66 ± 3.47

No. Mice = 3

Mean Weight = 19.58 grams ± 0.71 grams

Table 32

Distribution of Activity at 24 Hours Following I.V. Injection of ^{90}Y-2B8-MX-DTA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.55 ± 0.12	19.77 ± 2.42	30.77 ± 6.04
Heart	0.105 ± 0.01	4.44 ± 0.55	0.47 ± 0.08
Lung (2)	0.127 ± 0.02	8.78 ± 1.61	1.11 ± 0.21
Kidney (1)	0.139 ± 0.01	5.02 ± 0.52	0.69 ± 0.05
Liver	0.966 ± 0.09	8.62 ± 2.73	8.20 ± 1.97
Spleen	0.083 ± 0.01	6.75 ± 1.27	0.55 ± 0.064
Muscle	8.83 ± 0.69	0.692 ± 0.01	6.12 ± 0.52
Bone	3.31 ± 0.26	2.24 ± 0.31	7.47 ± 1.53
Skin	3.31 ± 0.26	3.33 ± 0.76	10.88 ± 1.76
GI Tract	2.89 ± 0.43	0.73 ± 0.09	1.02 ± 0.05
Urine			2.31
Feces			1.23
		Total:	73.52 ± 6.18%

No. Mice = 3

Mean Weight = 22.09 ± 1.73 gram

Table 33

Distribution of Activity at 48 Hours Following I.V. Injection of ^{90}Y-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.50 ± 0.14	14.97 ± 5.77	22.53 ± 8.48
Heart	0.104 ± 0.01	3.99 ± 1.43	0.415 ± 0.16
Lung (2)	0.122± 0.02	8.41 ± 1.57	1.04± 0.31
Kidney (1)	0.124 ± 0.01	3.99 ± 1.62	0.49 ± 0.19
Liver	0.966 ± 0.13	6.12 ± 3.21	5.69 ± 2.25
Spleen	0.079 ± 0.01	6.05 ± 2.38	0.46 ± 0.16
Muscle	8.59 ± 0.82	0.54 ±0.19	4.67±1.67
Bone	3.22 ± 0.31	2.07 ± 0.84	6.65 ± 2.56
Skin	3.22 ± 0.31	2.30 ± 0.70	7.34 ± 1.95
GI Tract	2.63 ± 0.40	0.652 ± 0.30	1.67 ± 0.64
Urine	--	--	2.83
Feces	--	--	2.06
		TOTAL	57.28 ± 17.60

No. Mice = 3

Mean Weight = 21.48 ± 2.05 grams

Table 34

Distribution of Activity at 72 Hours Following I.V. Injection of ^{90}Y-2B8-MX-DTPA Into Normal BALB/c Mice

Mean Values ± SD

Sample	Organ Weight Gram	% ID/ Gram	% ID per Organ
Blood	1.45 ± 0.07	15.87 ± 4.81	23.14 ± 7.26
Heart	0.093 ± 0.01	4.16 ± 1.27	0.392 ± 0.13
Lung (2)	0.123± 0.02	10.67 ± 3.79	1.30 ± 0.45
Kidney (1)	0.123 ± 0.01	4.79 ± 1.03	0.596 ± 0.16
Liver	0.876 ± 0.07	7.26 ± 1.79	6.39 ± 1.76
Spleen	0.081 ± 0.01	7.37 ± 2.34	0.584 ± 0.16
Muscle	8.30 ± 0.39	0.67 ± 0.13	5.58 ± 1.22
Bone	3.11 ± 0.15	2.58 ± 0.51	8.05 ± 1.76
Skin	3.11 ± 0.15	3.09 ± 0.82	9.66 ± 2.68
GI Tract	2.59 ± 0.20	0.79 ± 0.18	2.05 ± 0.53
Urine	--	--	3.56
Feces	--	--	2.82
		TOTAL	65.47 ± 14.0

No. Mice = 3

Mean Weight = 20.76 ± 0.97 grams

4. Dosimetry

The radiation absorbed doses for a "standard" 70 Kg human calculated for the ^{90}Y-labeled conjugate using the mouse biodistribution data (%ID/organ values in Tables 31-34) are presented in Table 35. These results are comparable to results obtained previously using ^{90}Y-labeled 2B8-MX-DTPA prepared using the HPLC radiolabeling method.

Table 35

Radiation Dosimetry Estimates Resulting from the Administration of Yttrium-[90] Labeled 2B8-MX Uniformly Distributed in Standard Man(70 Kg) and Based on Animal Distribution Data Over 72 Hours After Injection

AMOUNT OF ACTIVITY =		1000 MICROCURIES/PATIENT DOSE	
	RADS		RADS
ADRENALS	0.534	OVARIES	0.534
BLADDER WALL	0.534	PANCREAS	0.534
STOMACH WALL	0.534	SKELETON	
SMALL INTESTINE	1.158	CORTICAL BONE	1.466
UL INTEST. WALL	1.657	TRABECULAR BONE	1.466
LL INTEST. WALL	2.380	MARROW (RED)	4.452
KIDNEYS	7.015	MARROW (YELLOW)	2.096
LIVER	7.149	CARTILAGE	1.466
LUNGS	2.157	OTHER CONSTIT.	1.466
OTHER TISSUES		SKIN	6.603
MUSCLE	2.646	SPLEEN	4.973
ADIPOSE	2.646	TESTES	0.534
BLOOD	2.646	THYROID	0.534
BRAIN	2.112	UTERUS (NONGRVD)	0.767
HEART	2.646	TOTAL BODY	1.755

Ref: A Schema for Absorbed-dose Calculation for Biologically Distributed Radionuclides, MIRD J. of Nucl. Med./Suppl. #1, 2/68

Calculations Performed Using a Spreadsheet Template in Symphony (Lotus Development Corporation) and Created by Phillip L. Hagan, MS, Nuclear Medicine Service, VA Hospital, San Diego, CA 92161

5. Validation of Protocol for Preparation of Clinical Doses of Yttrium-[90]-2B8-MX-DTPA

A total of ten validation lots were prepared at MPI Pharmacy Services, Inc. The results of testing on each lot are summarized in Table 36. The mean value for each test result was calculated and standard deviations noted where appropriate. To evaluate the variability of the process due to different labeling times, lot #1 through #8 were prepared using a 10 min labeling time; lot #9 and #10 were prepared using a reaction time of 5 min. Based on the test results for the ten validation lots, release specifications were established. Release specifications are summarized in Table 37.

Table 36

Assay Results for the Ten Validation Lots of ^{90}Y-Labeled

2B8-MX-DTPA Prepared Using "Mix-&-Shoot"

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	mean
%immunoreactivity	72.8	93.3	71.7	70.2	60.6	68.2	79.5	72.4	88.2	68.5	74.5 ± 9.8
endotoxin (Eu/ml)	<0.125	<0.125	<0.125	<0.125	<0.125	<0.25	<0.25	<0.25	<0.125	<0.125	<0.162 ± 0.06
%radioincorporation	97.5	97.0	93.5	96.0	94.7	94.9	95.9	96.5	97.5	93.5	95.7 ± 1.4
antibody conc. (mg/ml)	0.122	0.102	0.088	0.128	0.134	0.119	0.093	0.088	0.111	0.096	0.108 ± 0.017
radioactivity (mCi/ml)	1.22	1.22	0.98	1.26	1.51	1.55	1.06	0.98	1.28	1.02	1.21 ± 0.21
specific act. (mCi/mg)	10.0	12.0	11.2	9.8	11.3	13.0	11.3	11.1	11.5	10.7	11.2 ±0.9
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7. The radiolabeling kit of claim 3, wherein the chelator is MX-DTPA.

8. The radiolabeling kit of claim 1, wherein the antibody is 2B8.

9. The radiolabeling kit of claim 8, wherein the chelator-conjugated antibody is 2B8-MX-DTPA.

10. The kit of claim 1 wherein the antibody conjugate is supplied at a concentration of about 0.5 to 30 mg/ml.

11. The radiolabeling kit of claim 5 wherein the sodium acetate solution is at a concentration of 10 to 1000 mM.

12. The radiolabeling kit of claim 11 wherein the sodium acetate solution is at a concentration of 50 mM.

13. The radiolabeling kit of claim 1 wherein the formulation buffer comprises a radioprotectant and non-protein-conjugated chelator.

14. The radiolabeling kit of claim 13 wherein the radioprotectant is selected from the group consisting of human serum albumin (HSA), ascorbate, ascorbic acid, phenol, sulfites, glutathione, cysteine, gentisic acid, nicotinic acid, ascorbyl palmitate, $HOP(:O)H_2$, glycerol, sodium formaldehyde sulfoxylate, $Na_2S_2O_5$, $Na_2S_2O_3$, and SO_2.

15. The radiolabeling kit of claim 14 wherein the radioprotectant is HSA.

16. The radiolabeling kit of claim 13 wherein the unconjugated chelator is DTPA or EDTA.

17. The radiolabeling kit of claim 15 wherein the HSA is at a concentration of about 1 to 25% (w/v).

18. The radiolabeling kit of claim 17 wherein the concentration of HSA is about 7.5% (w/v).

19. The radiolabeling kit of claim 14 wherein the radioprotectant is ascorbate.

20. The radiolabeling kit of claim 19 wherein the ascorbate is at a concentration of about 1 to 100 mg/ml.

21. The radiolabeling kit of claim 1 wherein the radioisotope is ^{111}In chloride.

22. The radiolabeling kit of claim 1 wherein the radioisotope is ^{90}Y chloride.

23. A formulation buffer for administering a radiolabeled chelator-conjugated antibody to a patient comprising:

(i) a physiological salt solution;

(ii) a radioprotectant; and

(iii) non-protein conjugated chelator.

24. The formulation buffer of claim 23 wherein the radioprotectant is selected from the group consisting of HSA, ascorbate, ascorbic acid, phenol, sulfites, glutathione, cysteine, gentisic acid, nicotinic acid, ascorbyl palmitate, HOP(:O)H_2, glycerol, sodium formaldehyde sulfoxylate, $Na_2S_2O_5$, $Na_2S_2O_3$, and SO_2.

25. The formulation buffer of claim 23 wherein the chelator is DTPA.

26. The formulation buffer of claim 24 wherein the radioprotectant is HSA.

27. The formulation buffer of claim 24 wherein the radioprotectant is ascorbate.

28. The formulation buffer of claim 26 wherein the concentration of human serum albumin is about 1 to 25% (w/v).

29. The formulation buffer of claim 28 wherein the HSA concentration is about 7.5%.

30. The formulation buffer of claim 25 wherein the concentration of DTPA is about 1 mM.

31. The formulation buffer of claim 27 wherein the concentration of ascorbate is about 1 to 100 mg/mL.

32. A method for radiolabeling a chelator-conjugated anti-CD20 antibody for administration to a patient comprising

(i) mixing chelator-conjugated antibody with a solution containing a radiolabel;

(ii) incubating the mixture for an appropriate amount of time at appropriate temperature; and

(iii) diluting the labeled antibody to an appropriate concentration in formulation buffer, such that said radiolabeled antibody may be administered directly to the patient without further purification.

33. The method of claim 32 wherein the antibody is a chimeric anti-CD20 antibody.

34. The method of claim 32 wherein the anti-CD20 antibody is 2B8-MX-DTPA.

35. The method of claim 32 wherein the formulation buffer contains physiological saline, a radioprotectant, and unconjugated chelator.

36. The method of claim 35 wherein the radioprotectant is selected from the group consisting of human serum albumin (HSA), ascorbate, ascorbic acid, phenol, sulfites, glutathione, cysteine, gentisic acid, nicotinic acid, ascorbyl palmitate, $HOP(:O)H_2$, glycerol, sodium formaldehyde sulfoxylate, $Na_2S_2O_5$, $Na_2S_2O_3$, and SO_2.

37. The method of claim 35 wherein the unconjugated chelator is DTPAor EDTA.

38. The method of claim 32 wherein the solution containing the radiolabel is adjusted to a pH of about 3 to 6 before it is mixed with the chelator-conjugated antibody.

39. The method of claim 38 wherein the pH is adjusted with a low metal sodium acetate solution.

40. The method of claim 39 wherein the sodium acetate is at a concentration of about 10 to 1000 mM.

41. The method of claim 32 wherein the radiolabel is ^{111}In chloride.

42. The method of claim 41 wherein the volume quantity of ^{111}In chloride used is about 4-6 mCi divided by the radioactivity concentration at the time of labeling.

43. The method of claim 41 wherein about 1 ml of chelator-conjugated antibody at a concentration of about 0.5 to 30 mg/ml is mixed with the radiolabel solution.

44. The method of claim 43 wherein the mixture is incubated for between about 30 seconds and 60 minutes.

45. The method of claim 44 wherein formulation buffer is added in an amount necessary to achieve a total final volume of about 10 ml to about 50 ml.

46. The method of claim 32 wherein the radiolabel is ^{90}Y chloride.

47. The method of claim 46 wherein the volume quantity of ^{90}Y chloride used is between about 5 to 100 mCi divided by the radioactivity concentration at the time of labeling.

48. The method of claim 47 wherein the volume quantity of ^{90}Y chloride used is about 45 mCi divided by the radioactivity concentration at the time of labeling.

49. The method of claim 46 wherein about 1 to 2 ml of MX-DTPA-conjugated antibody at a concentration of about 0.5 to 30 mg/ml is mixed with the radiolabel solution.

50. The method of claim 49 wherein the mixture is incubated for a time between about 30 seconds to 60 minutes.

51. The method of claim 50 wherein formulation buffer is added in an amount necessary to achieve a total final volume of about 10 ml to about 50 ml.

52. A binding assay and radiolabeling kit for radiolabeling an anti-CD20 antibody and determining the percent binding of the radiolabeled antibody to its target cell before administering the antibody to a patient, comprising the following components:

(i) at least one vial of fixed or lyophilized antigen-positive cells;
(ii) a vial containing a chelator-conjugated antibody;
(iii) a vial containing formulation buffer; and
(iv) instructions for radiolabeling the antibody,

wherein said vial components are supplied in such an amount and at such a concentration that when they are combined with a radiolabel according to the kit instructions, no further purification of the labeled antibody is required before administration to said patient.

53. The binding assay and radiolabeling kit of claim 52, wherein said antibody is a chimeric anti-CD20 antibody.

54. The binding assay and radiolabeling kit of claim 52 further comprising a vial containing a buffer for adjusting the pH of the radiolabel.

55. The binding assay and radiolabeling kit of claim 52 wherein the formulation buffer is phosphate buffered saline comprising a radioprotectant and unconjugated chelator.

56. The binding assay and radiolabeling kit of claim 52, wherein the antigen positive cells are CD20-positive cells.

57. The binding assay and radiolabeling kit of claim 56 wherein the CD20-positive cells are SB cells (ATCC # CCL 120).

58. The binding assay and radiolabeling kit of claim 52 further comprising at least one vial of antigen-negative cells.

59. The binding assay and radiolabeling kit of claim 58, wherein said antigen-negative cells are CD20-negative cells.

60. The binding assay and radiolabeling kit of claim 59 wherein said CD20-negative cells are HSB cells (ATCC # CCL120.1).

61. A binding assay kit for determining the percent binding of a radiolabeled anti-CD20 antibody to its target cell comprising at least one vial of fixed or lyophilized antigen-positive cells.

62. The binding assay kit of claim 61, further comprising a control anti-CD20 antibody.

63. The binding assay kit of claim 62 wherein the CD20-positive cells are SB cells (ATCC # CCL 120).

64. The binding assay kit of claim 61 further comprising antigen-negative cells.

65. The binding assay kit of claim 64, wherein said cells are CD20-negative.

66. The binding assay kit of claim 65 wherein said CD20-negative cells are HSB cells (ATCC # CCL120.1).

67. A binding assay for determining the percent binding of a radiolabeled antibody to its target cell , comprising:

(i) mixing and incubating at least one aliquot of a radiolabeled antibody with at least one aliquot of antigen-positive cells;

(ii) mixing and incubating at least one aliquot of a radiolabeled antibody identical to the aliquot of step (i) with at least one aliquot of dilution buffer of the same volume as the aliquot of antigen-positive cells in step (i) as a control;

(iii) pelleting the cells by centrifugation;

(iv) measuring the radioactivity in the supernatant of the pelleted cells and the control; and

(v) comparing the quantity of radioactivity in the cell supernatant to the quantity of radioactivity in the control.

68. The binding assay of claim 67 wherein said antibody is a CD20 antibody.

69. The binding assay of claim 68 wherein the anti-CD20 antibody is 2B8.

70. The binding assay of claim 67 wherein said antigen is CD20.

71. The binding assay of claim 70 wherein said CD20 positive cells are SB cells (ATCC # CCL 120).

72. The binding assay of claim 67 further comprising

(i) mixing at least one aliquot of the radiolabeled antibody with at least one aliquot of antigen-negative cells;

(ii) pelleting the antigen-negative cells by centrifugation;

(iv) measuring the radioactivity in the supernatant of the antigen-negative pelleted cells; and

(v) comparing the quantity of radioactivity in the antigen-negative cell supernatant to the quantity of radioactivity in the supernatant of the antigen-positive cell supernatant and the control.

73. The binding assay of claim 72 wherein said antigen negative cells are CD20-negative.

74. The binding assay of claim 73 wherein said CD20 negative cells are HSB cells (ATCC # CCL 120.1).

75. A binding assay for determining the percent binding of a radiolabeled antibody to its target cell , comprising:

(i) mixing at least one aliquot of a radiolabeled antibody with at least one aliquot of antigen-positive cells;

(ii) mixing at least one aliquot of the radiolabeled antibody identical to the aliquot of step (i) with at least one aliquot of dilution buffer of the same volume as the aliquot of antigen-positive cells in step (i) as a control;

(iii) pelleting the cells by centrifugation;

(iv) measuring the radioactivity in the supernatant of the pelleted cells and the control; and

(v) comparing the quantity of radioactivity in the cell supernatant to the quantity of radioactivity in the control;

wherein said assay is performed using the binding assay and radiolabeling kit of claim 52.

76. A binding assay for determining the percent binding of a radiolabeled antibody to its target cell , comprising:

(i) mixing at least one aliquot of a radiolabeled antibody with at least one aliquot of antigen-positive cells;

(ii) mixing at least one aliquot of a radiolabeled antibody identical to the aliquot of step (i) with at least one aliquot of dilution buffer of the same volume as the aliquot of antigen-positive cells in step (i) as a control;

(iii) pelleting the cells by centrifugation;

(iv) measuring the radioactivity in the supernatant of the pelleted cells and the control; and

(v) comparing the quantity of radioactivity in the cell supernatant to the quantity of radioactivity in the control;

wherein said assay is performed using the binding assay kit of claim 61.

77. A competitive binding assay for assessing affinity of a test antibody to a target cell, comprising

(i) preparing a ruthenium-labeled control antibody;

(ii) incubating increasing amounts of test antibody and increasing amounts of unlabeled control antibody with a fixed concentration of fixed, fresh or lyophilized antigen-positive cells and a trace amount of ruthenium-labeled antibody wherein each separate concentration of test antibody and each separate concentration of control antibody are in separate tubes, respectively;

(iii) determining the quantity of binding in each reaction tube based on relative electrochemiluminescence (ECL) using ORIGEN instrumentation; and

(iv) calculating the average affinity value of the test antibody.

78. The competitive binding assay of claim 77 wherein the control and test antibodies are anti-CD20 antibodies.

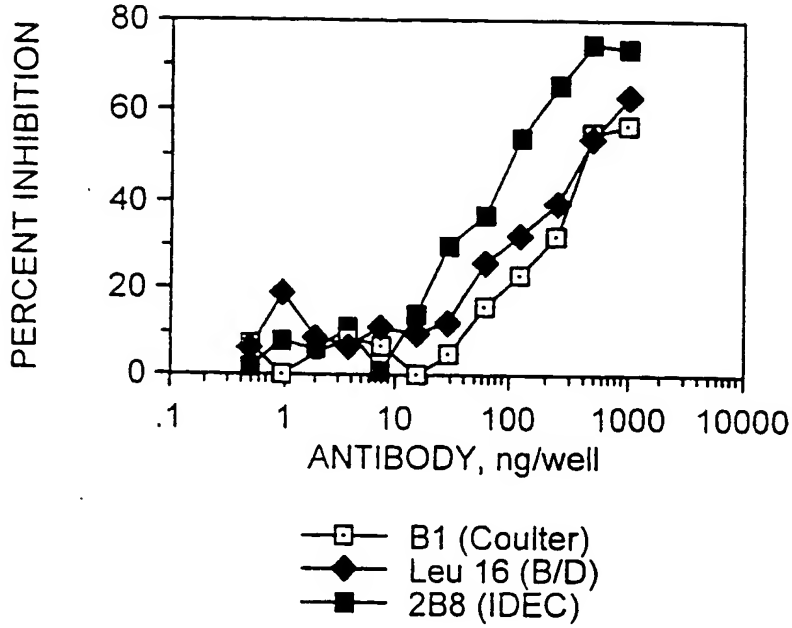
79. A competitive binding assay of claim 77 wherein the antigen-positive cells as CD20-positive.

80. The competitive binding assay of claim 79 wherein the antigen-positive cells are SB cells (ATCC # CCL 120).

81. The radiolabeling kit of claim 1 further comprising a vial of chimeric anti-CD20 antibody to be administered in a combined therapeutic regimen prior to or subsequent to the radiolabeled antibody.

82. The binding assay and radiolabeling kit of claim 52, further comprising a vial of chimeric anti-CD20 antibody to be administered in a combined therapeutic regimen prior to or subsequent to the radiolabeled antibody.

FIG. 1



80
60
40
20
0
PERCENT INHIBITION
.1
1
10
100
1000
10000
ANTIBODY, ng/well
B1 (Coulter)
Leu 16 (B/D)
2B8 (IDEC)

FIG. 2

FACS Analysis of Binding of 2B8 to CD20 Antigen Present on Human B-Cells

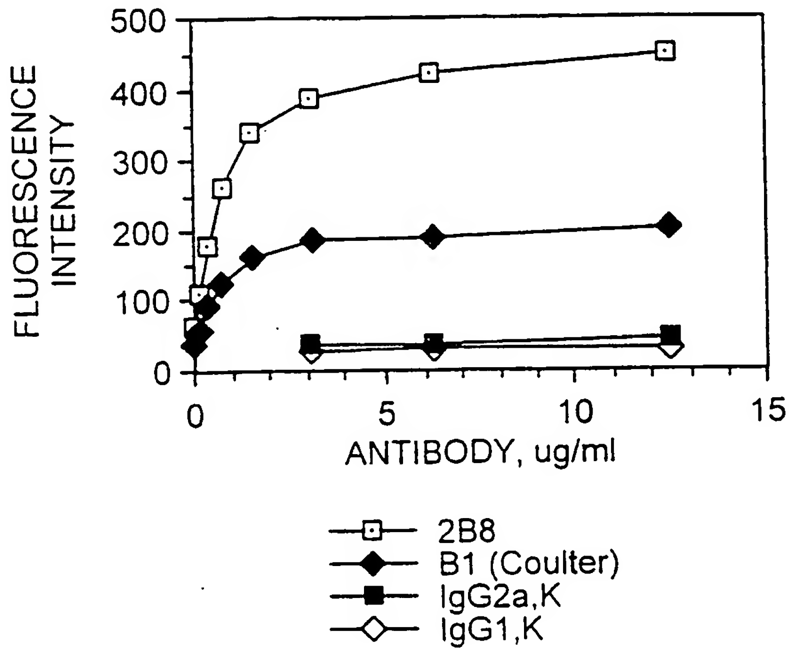


FIG. 3

Scatchard Analysis of Binding of 2B8 to B-Cells

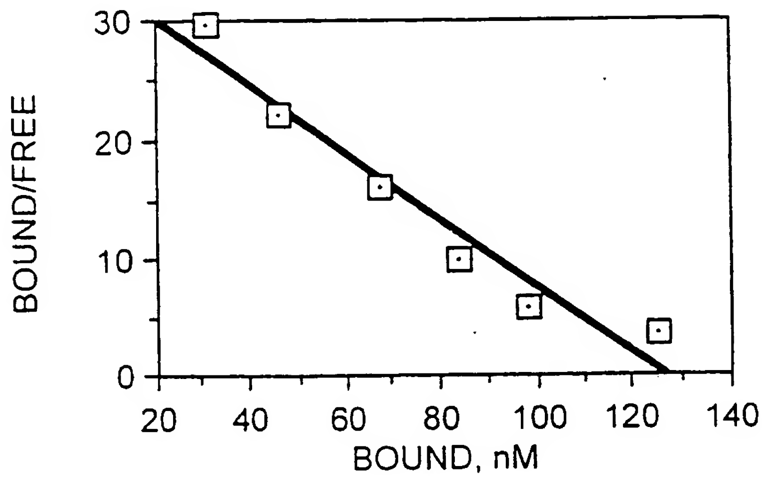


FIG. 4

Inhibition of Radiolabeled B1 Anti-CD20 Mab by Unlabeled B1, 2B8 and MX-DTPA Conjugated 2B8

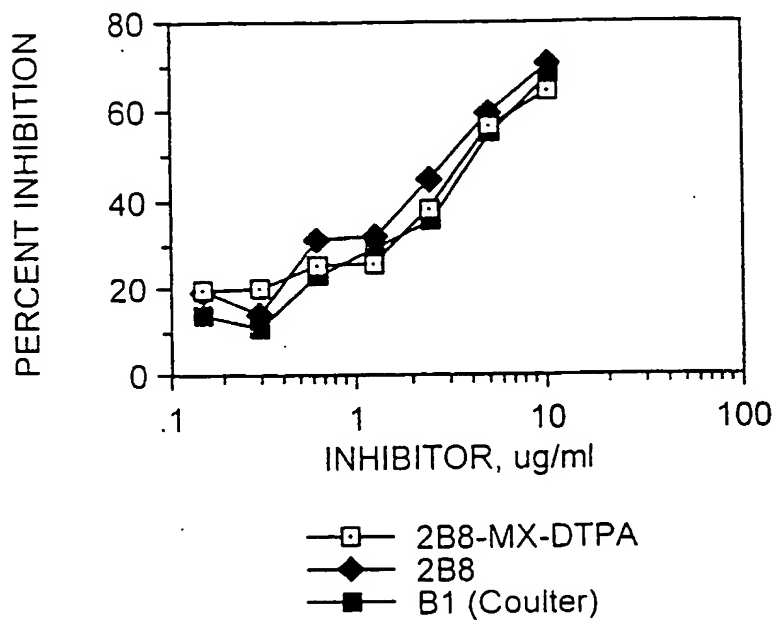


FIG. 5A

Immunoreactivity of 2B8 During Incubation at 4° and 30° C

2B8, 2B8-MX-DTPA Immunoreactivity Week 1

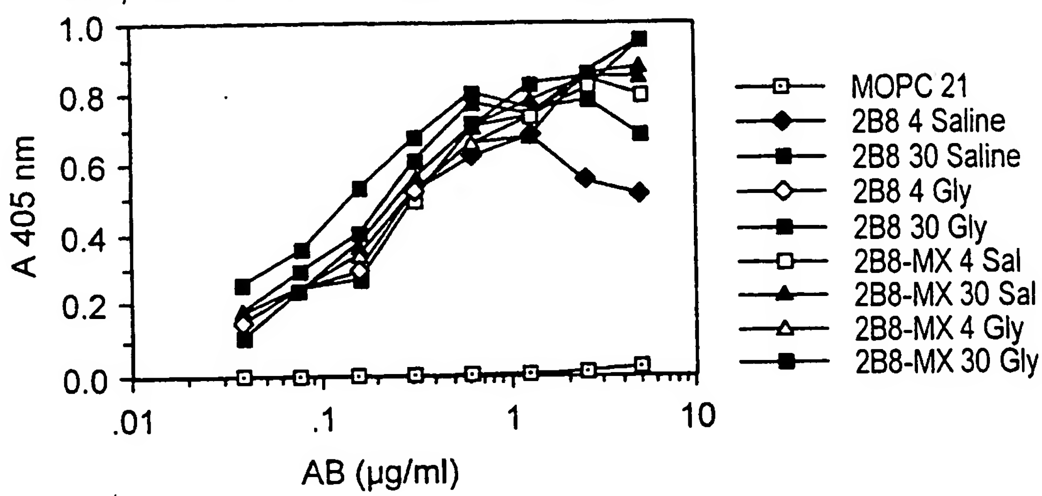


FIG. 5B

Immunoreactivity of 2B8 During Incubation at 4° and 30° C

2B8, 2B8-MX-DTPA Immunoreactivity Week 6

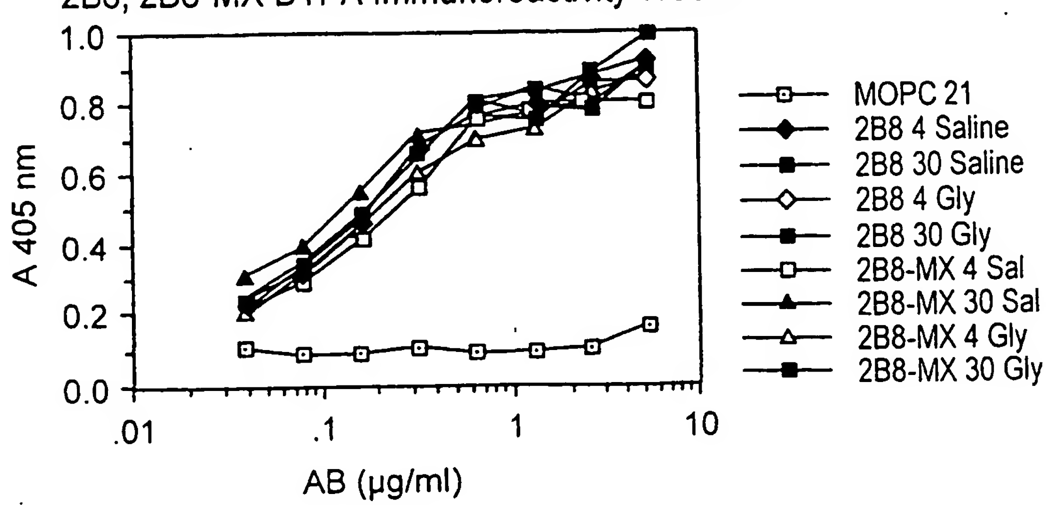


FIG. 5C

Immunoreactivity of 2B8 During Incubation at 4° and 30° C

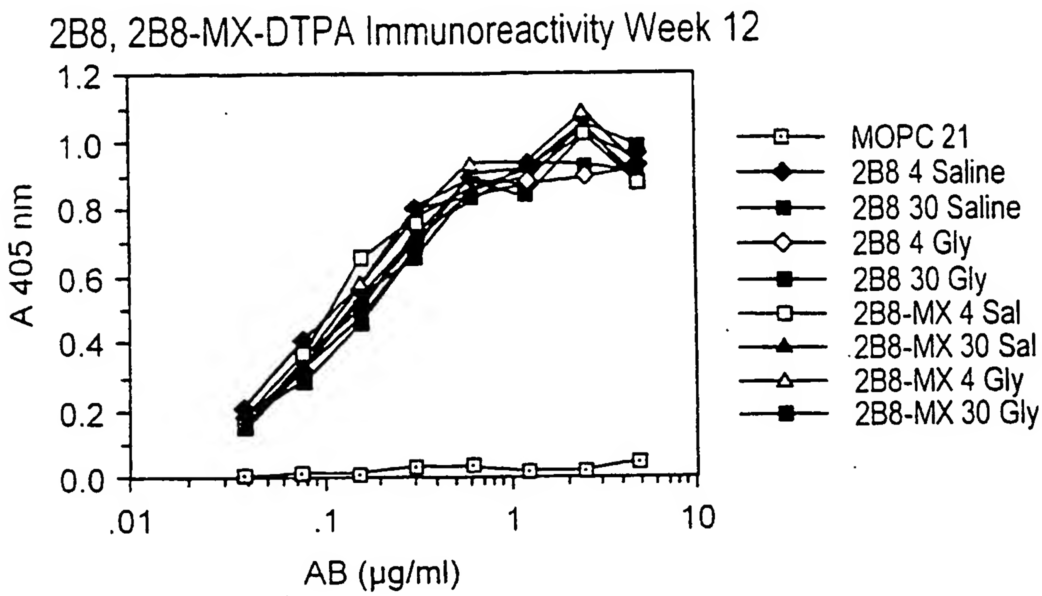


FIG. 6A

Binding of ^{111}In-Labeled 2B8-MX-DTPA to CD-20 Positive Human Cells

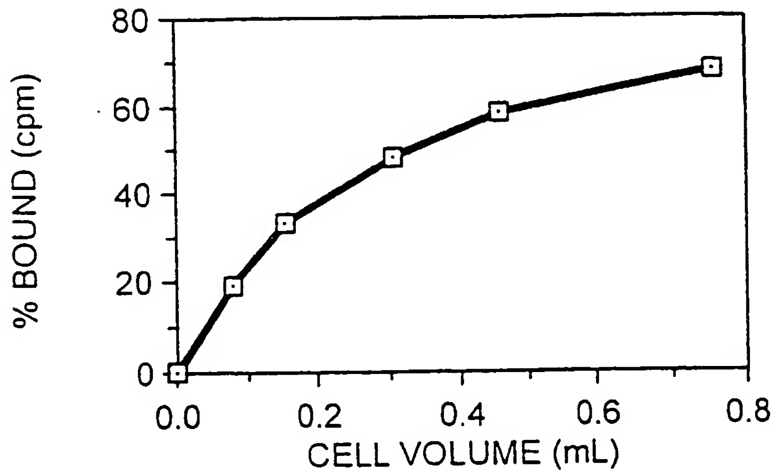


FIG. 6B

Binding of ^{111}In-Labeled 2B8-MX-DTPA to CD-20 Positive Human Cells

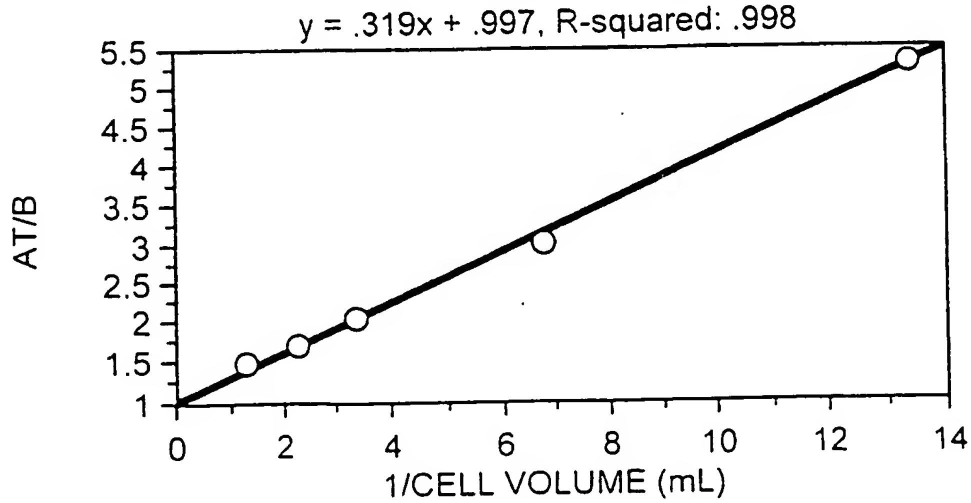


FIG. 7

In Vitro Stability of ^{90}Y-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin and DTPA

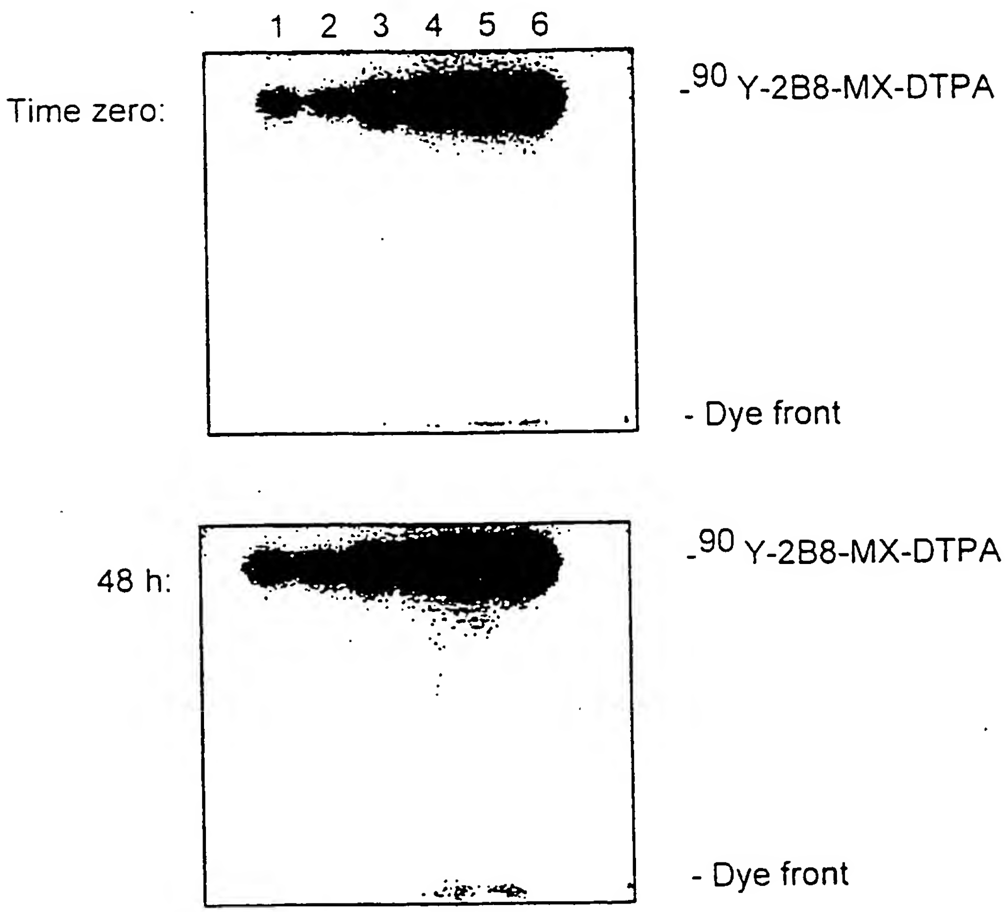


FIG. 8

In Vitro Stability of ^{90}Y-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin and DTPA

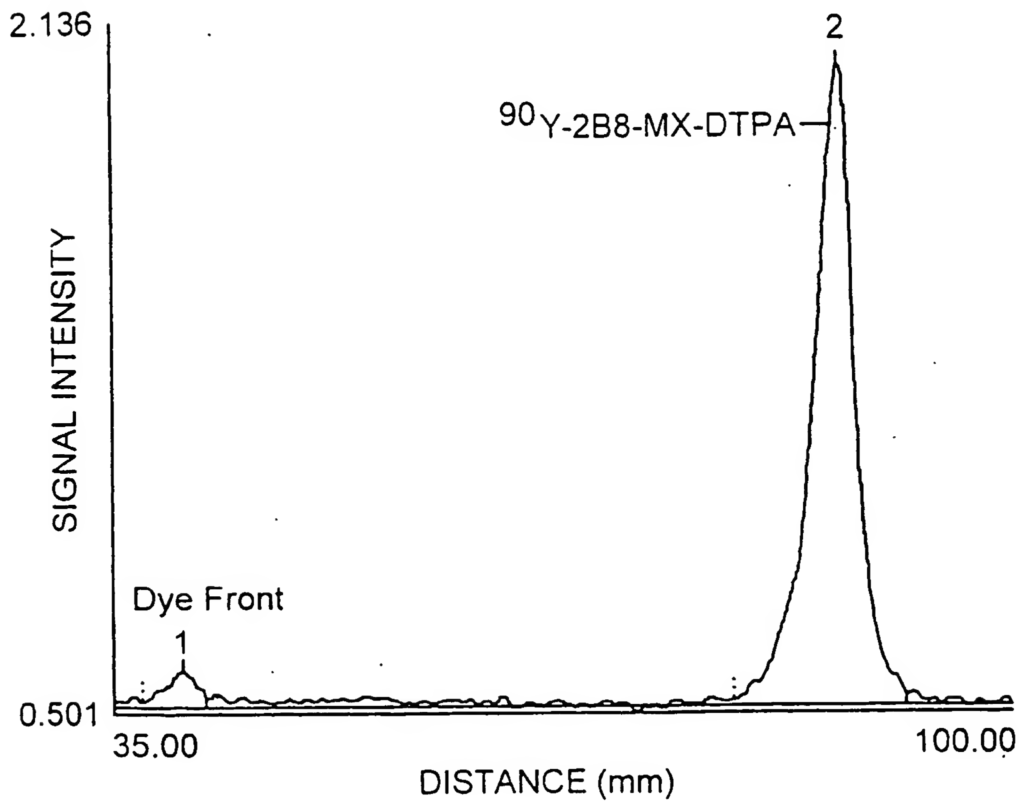


FIG. 9

In Vitro Stability of ^{90}Y-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin and DTPA

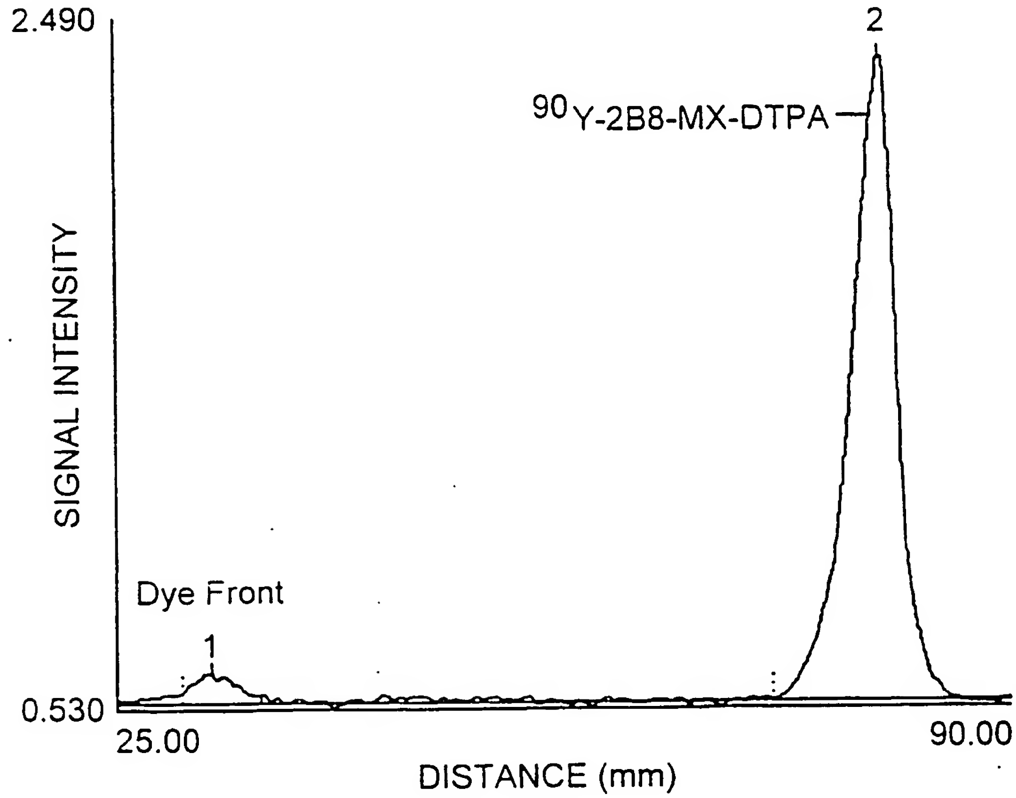


FIG. 10

In Vitro Stability of ^{111}In-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin

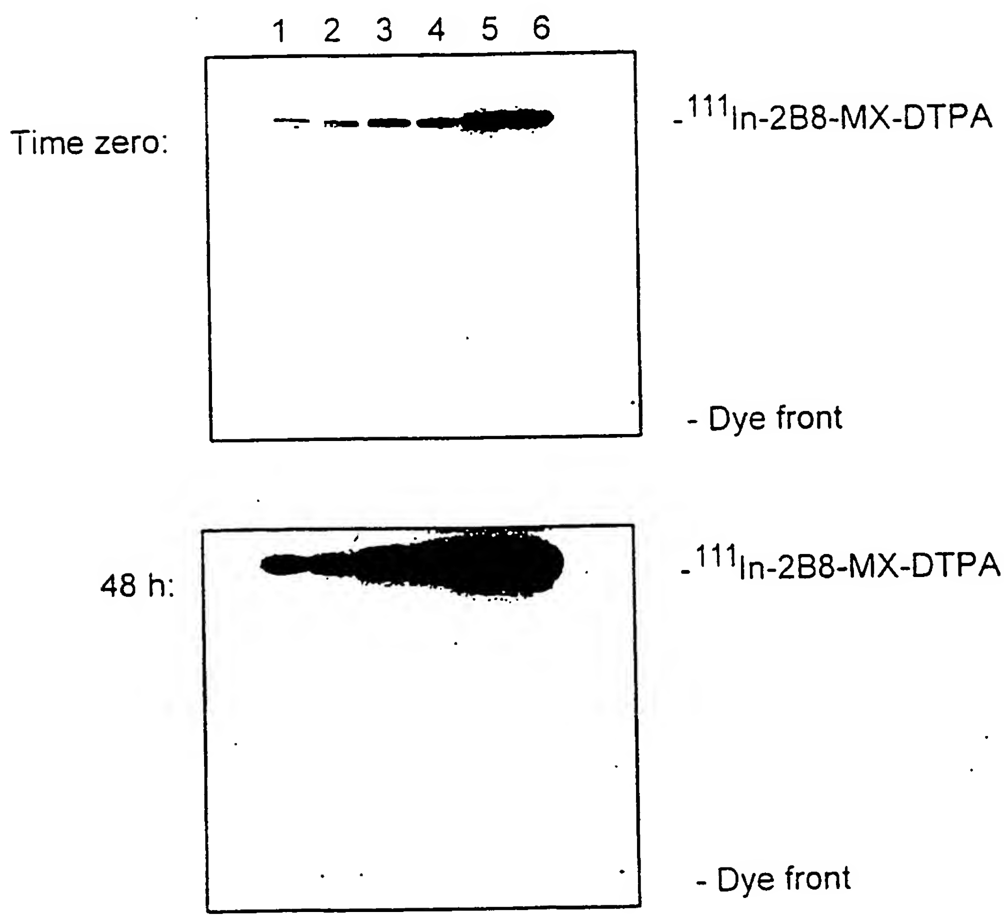


FIG. 11

In Vitro Stability of ^{111}In-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin

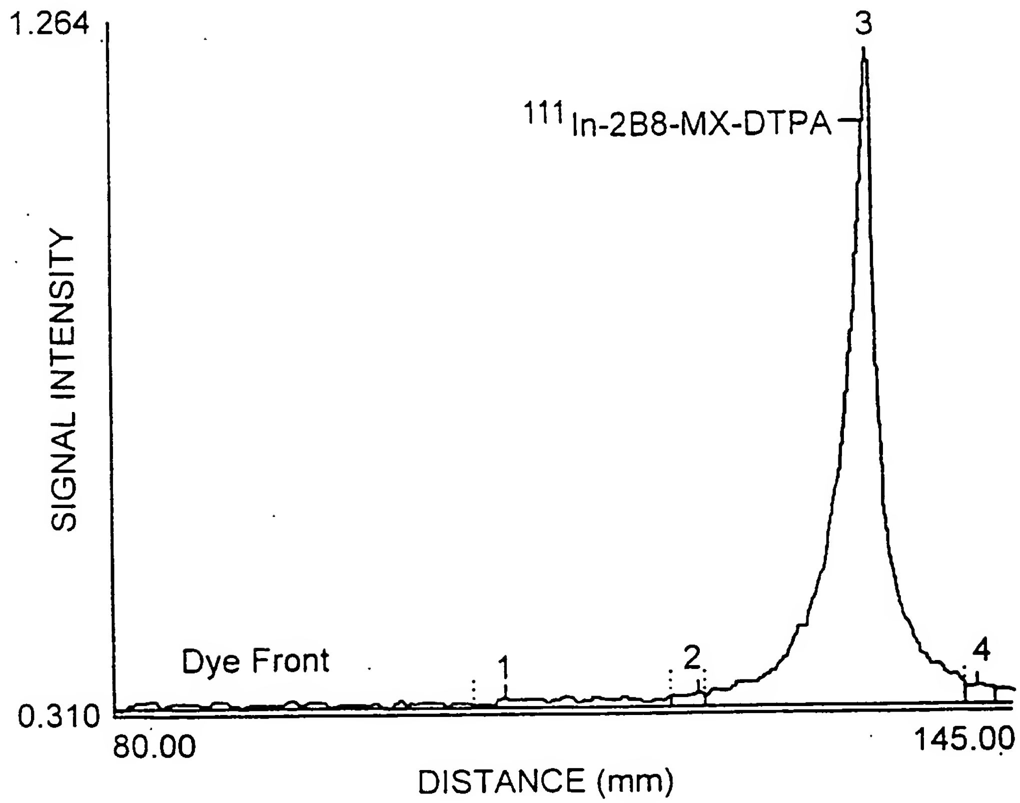


FIG. 12

In Vitro Stability of ^{111}In-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin

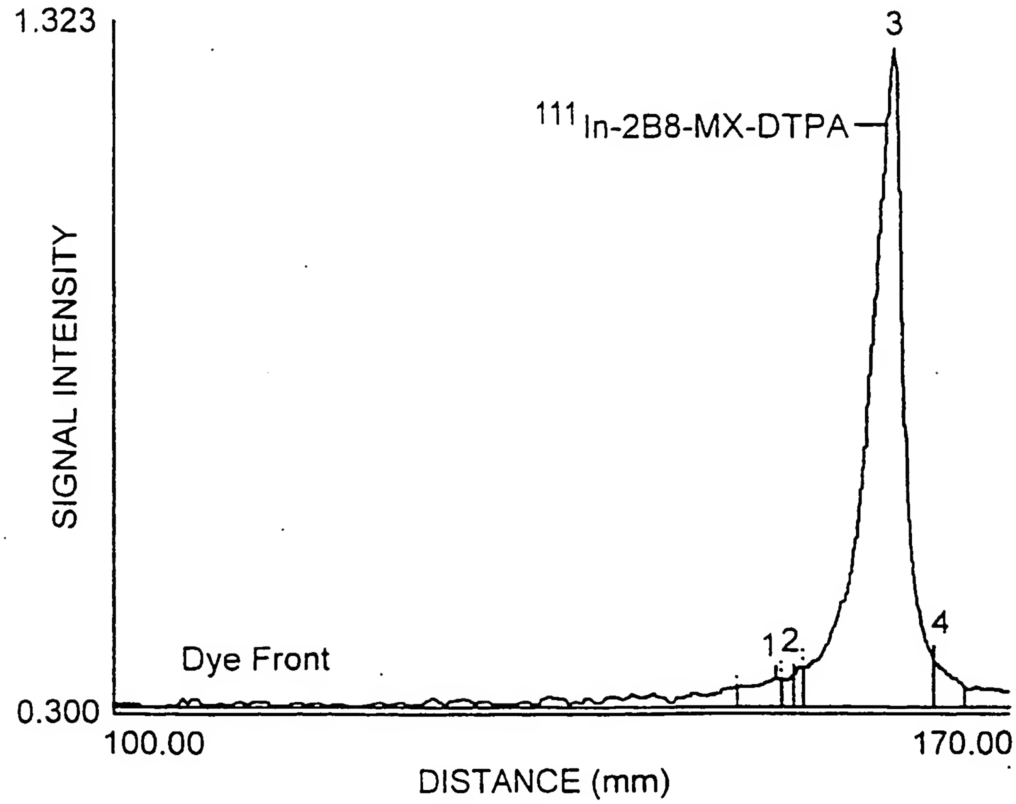


FIG. 13

In Vitro Stability of ^{90}Y-Labeled 2B8-MX-DTPA Incubated in Human Serum

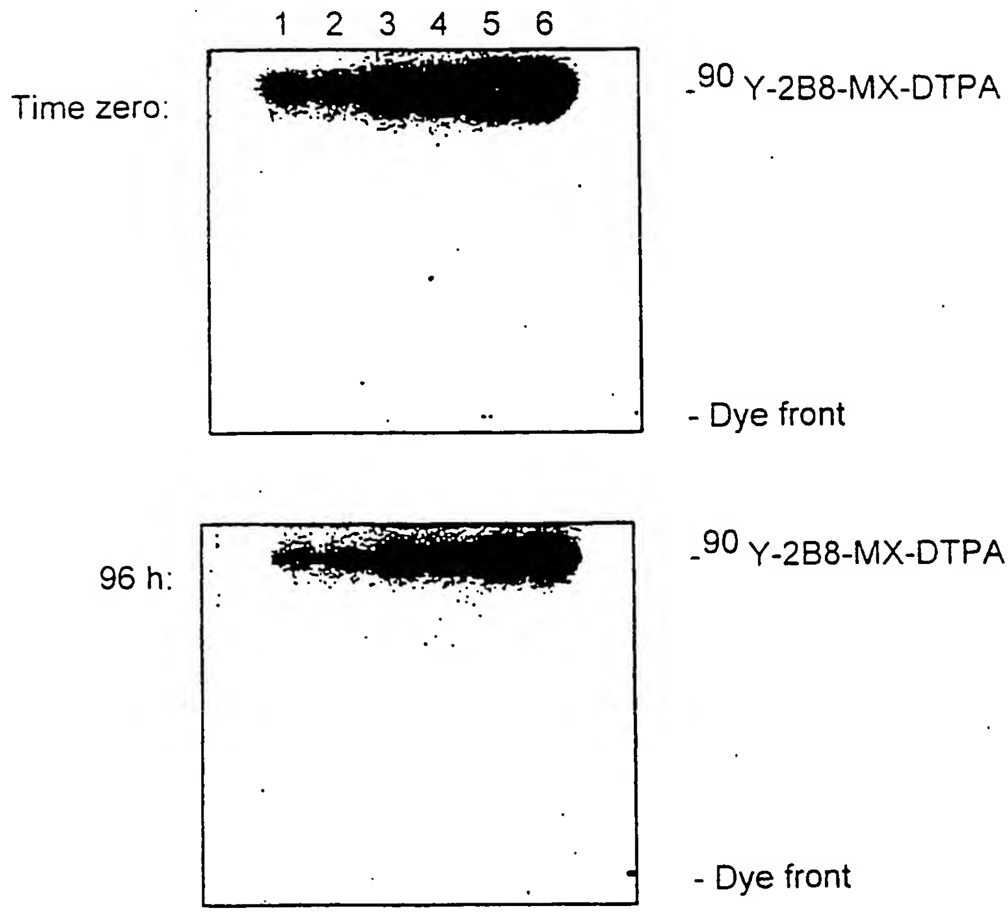


FIG. 14

In Vitro Stability of ^{90}Y-Labeled
2B8-MX-DTPA Incubated in Human Serum

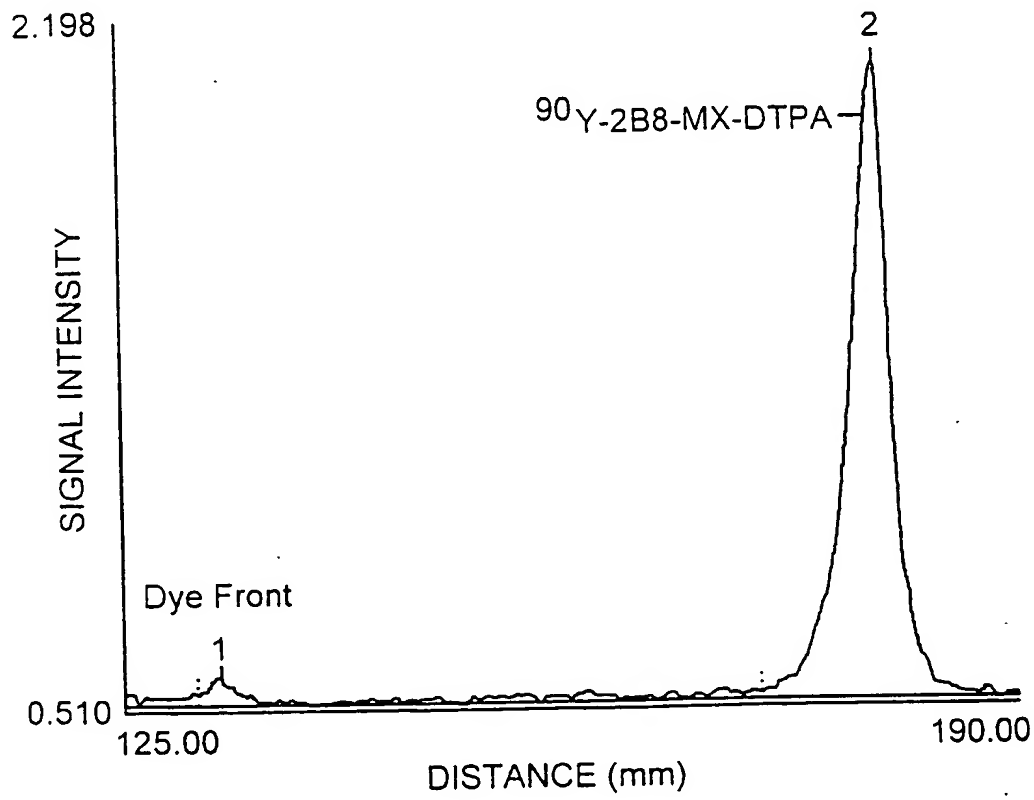


FIG. 15

In Vitro Stability of ^{90}Y-Labeled
2B8-MX-DTPA Incubated in Human Serum

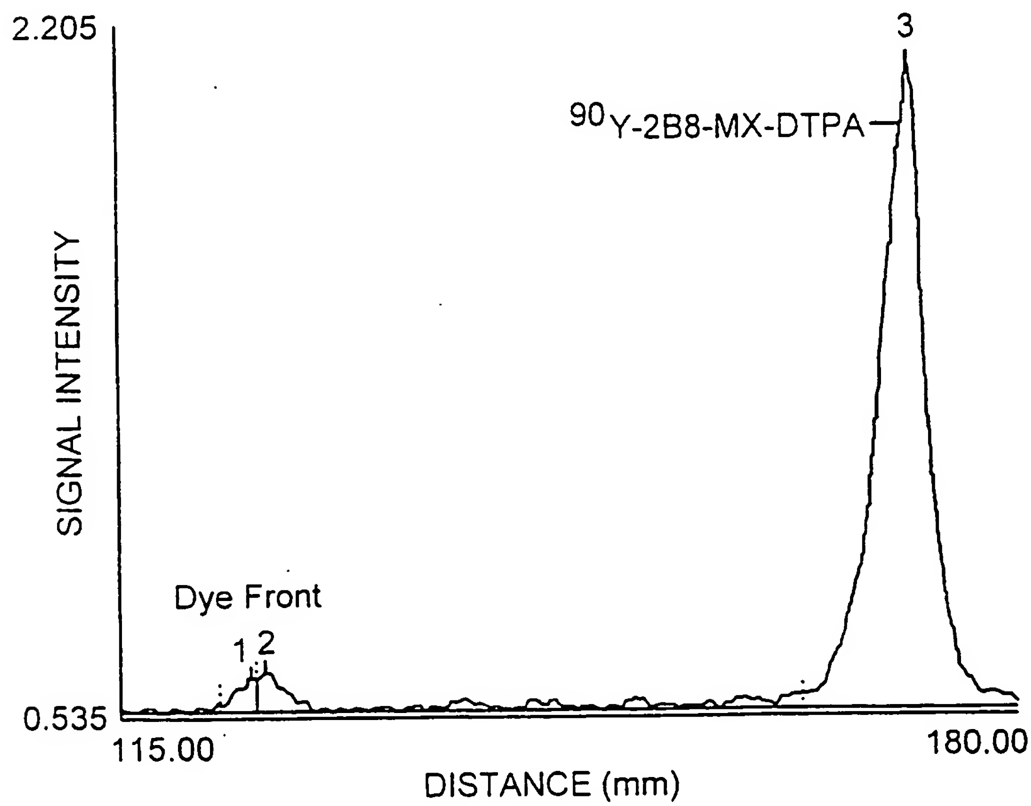


FIG. 16

In Vitro Stability of ^{111}In-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin

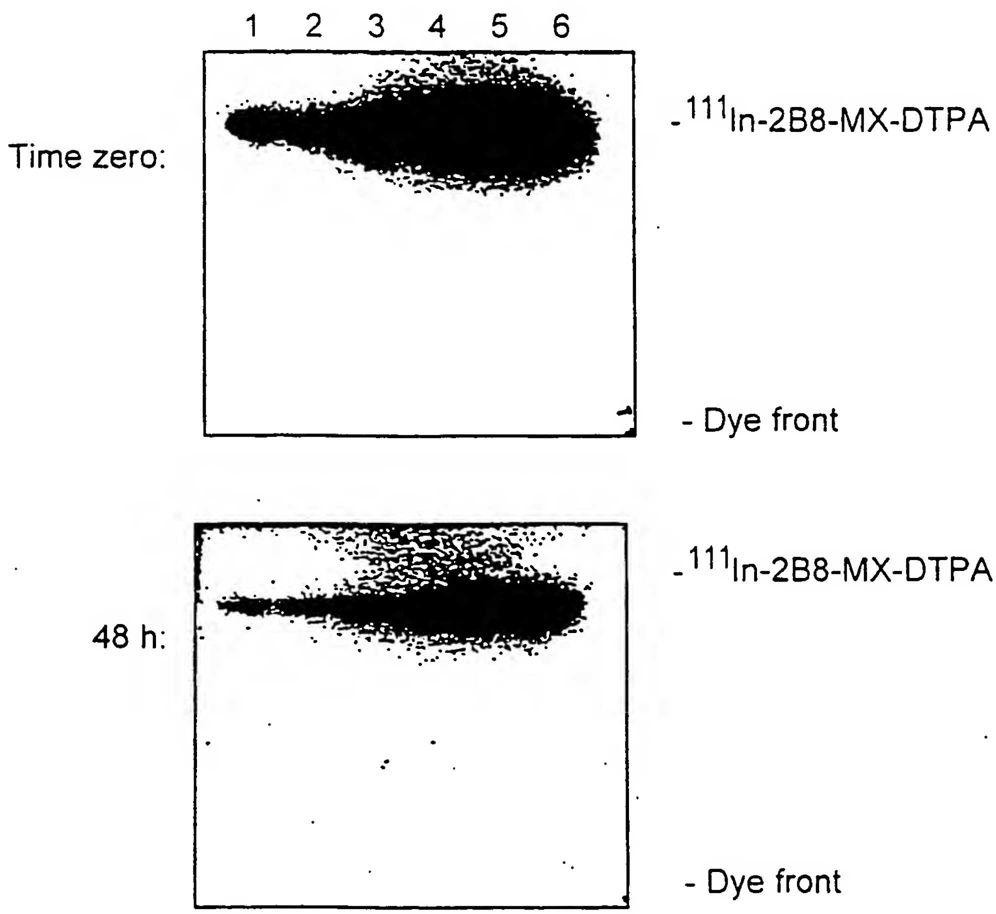


FIG. 17

In Vitro Stability of ^{111}In-Labeled 2B8-MX-DTPA Incubated in Human Serum

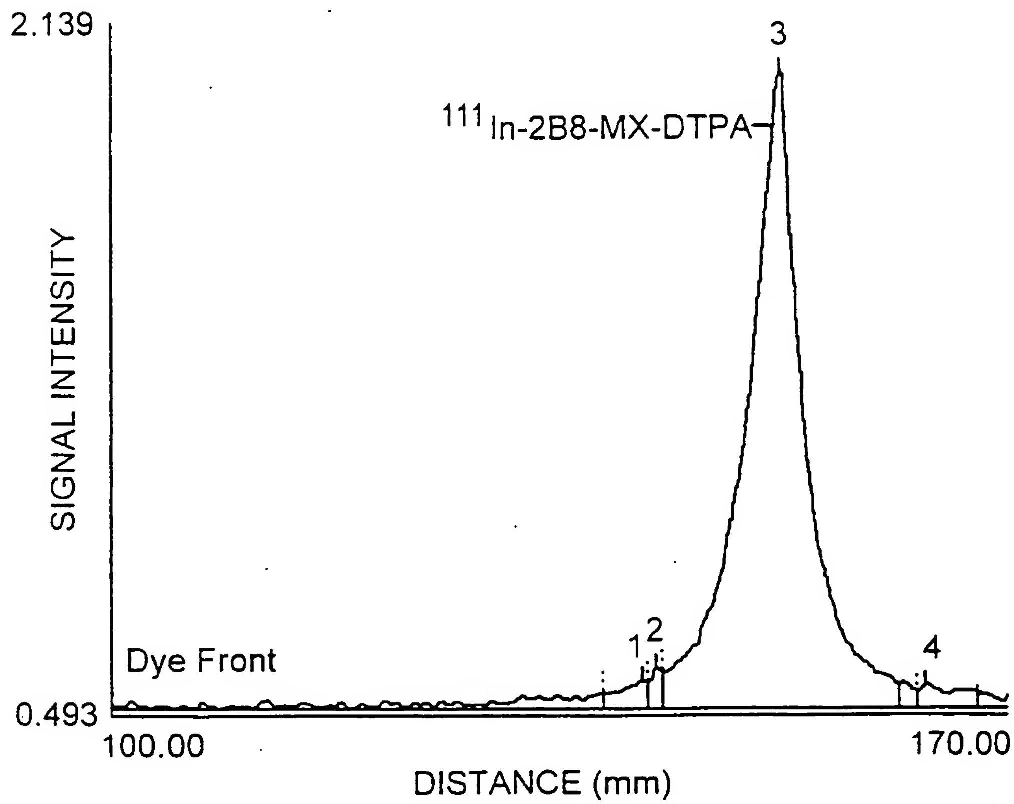


FIG. 18

In Vitro Stability of ^{111}In-Labeled
2B8-MX-DTPA Incubated in Human Serum

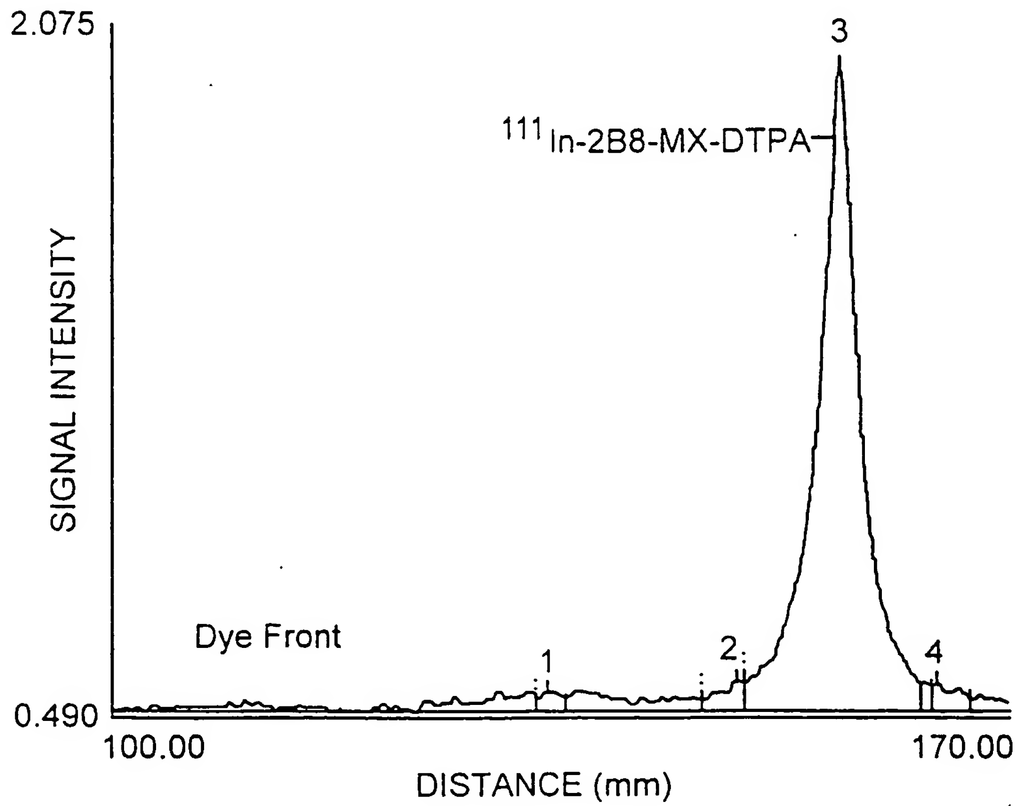
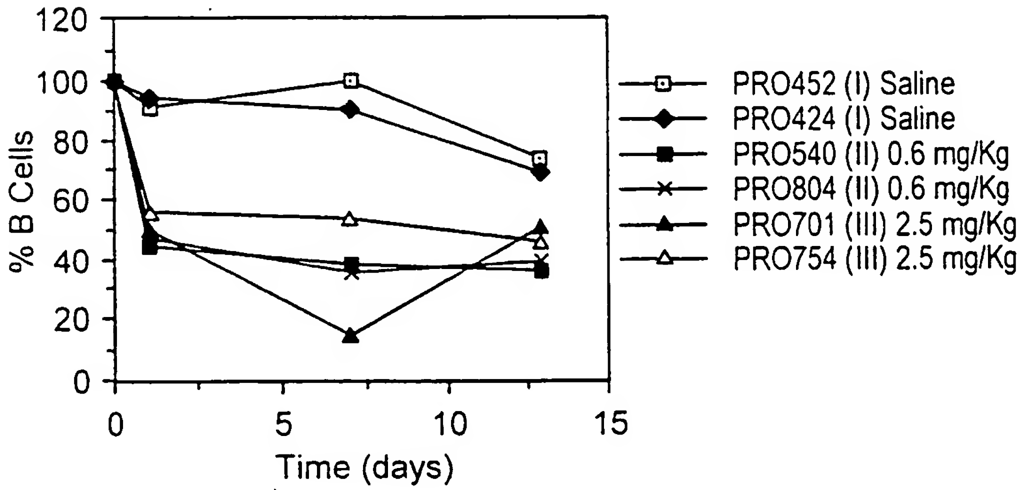


FIG. 19

Effect of 2B8 Infusion of B-lymphocyte Levels in Cynomolgus Monkeys, Day 0 through Day 13



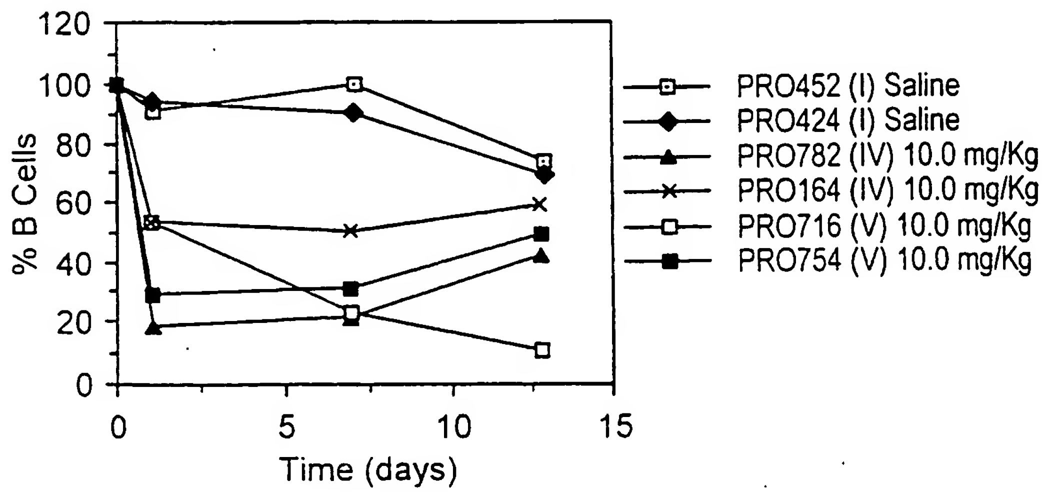


FIG. 20

Recovery of Circulating B-Cell Levels in Cynomolgus Monkeys Injected with Murine Monoclonal Anti-CD20 Antibody 2B8

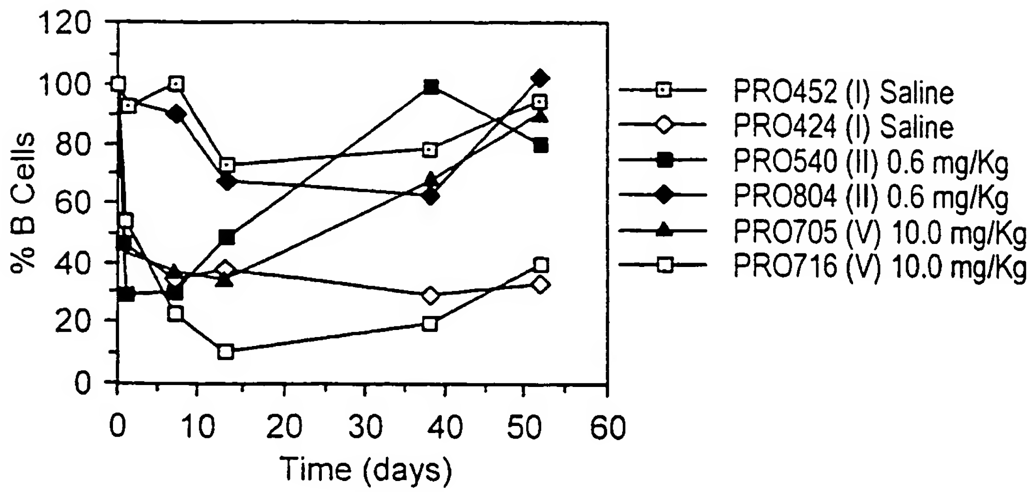


FIG. 21

Effect of 2B8-MX-DTPA on Circulating B-Cells in Cynomolgus Monkeys

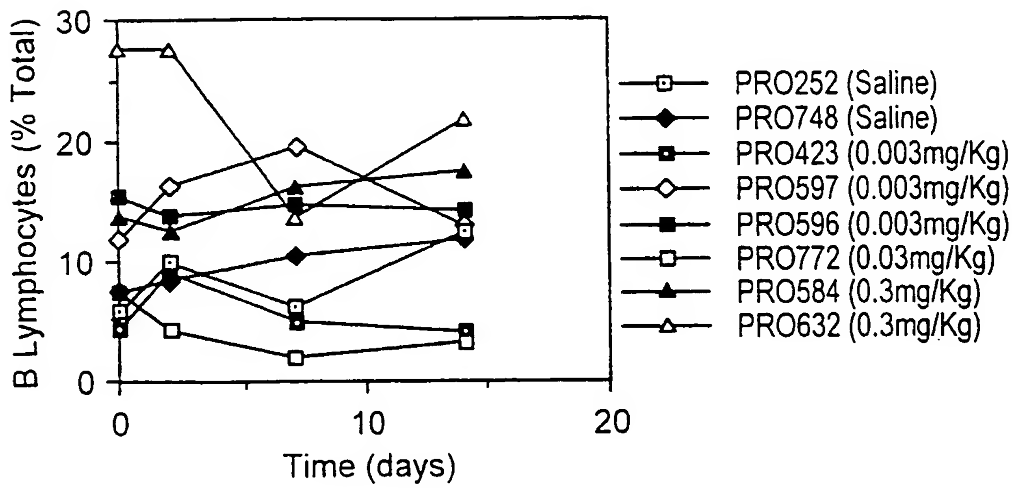


FIG. 22A

Clearance of 2B8 from Cynomolgus Monkeys Following a Single Injection of 10 mg/kg

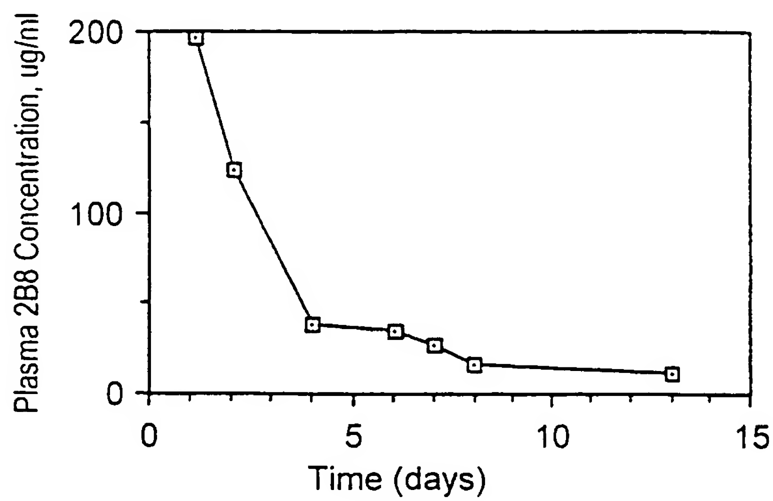


FIG. 22B

Blood Clearance of 2B8, 2B8-MX-DTPA from BALB/c Mice

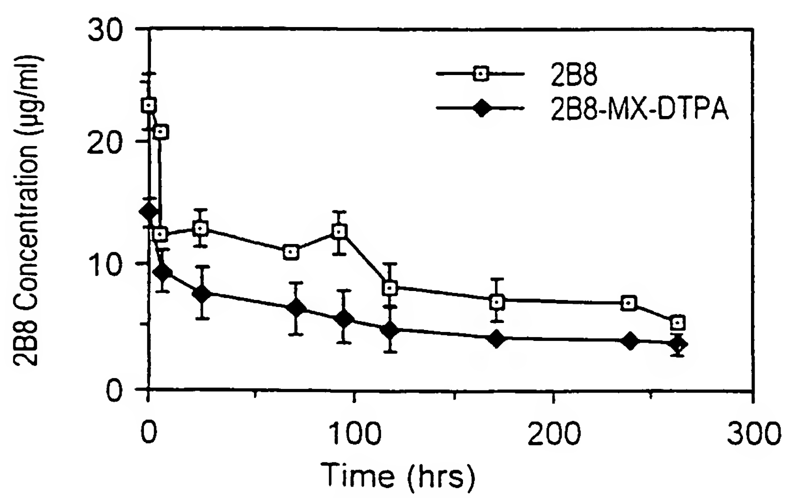


FIG. 23

[111]-In-2B8-MX-DTPA Biodistribution

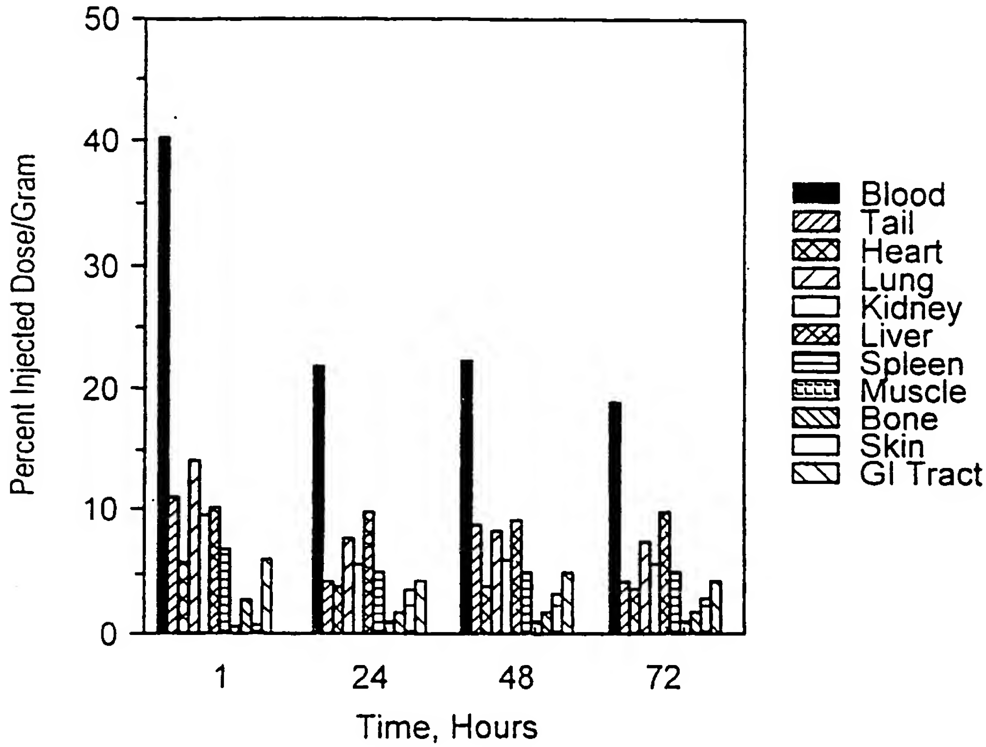


FIG. 24

[90]-Y-2B8-MX-DTPA Biodistribution

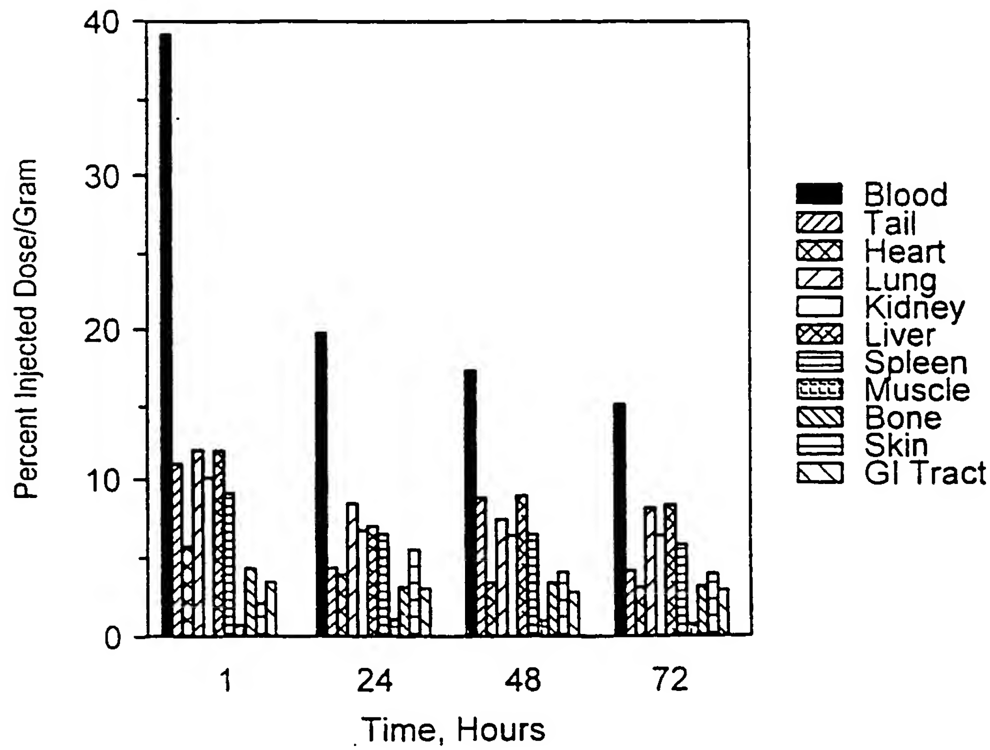


FIG. 25

111 In-2B8-MX-DTPA Tumor Localization

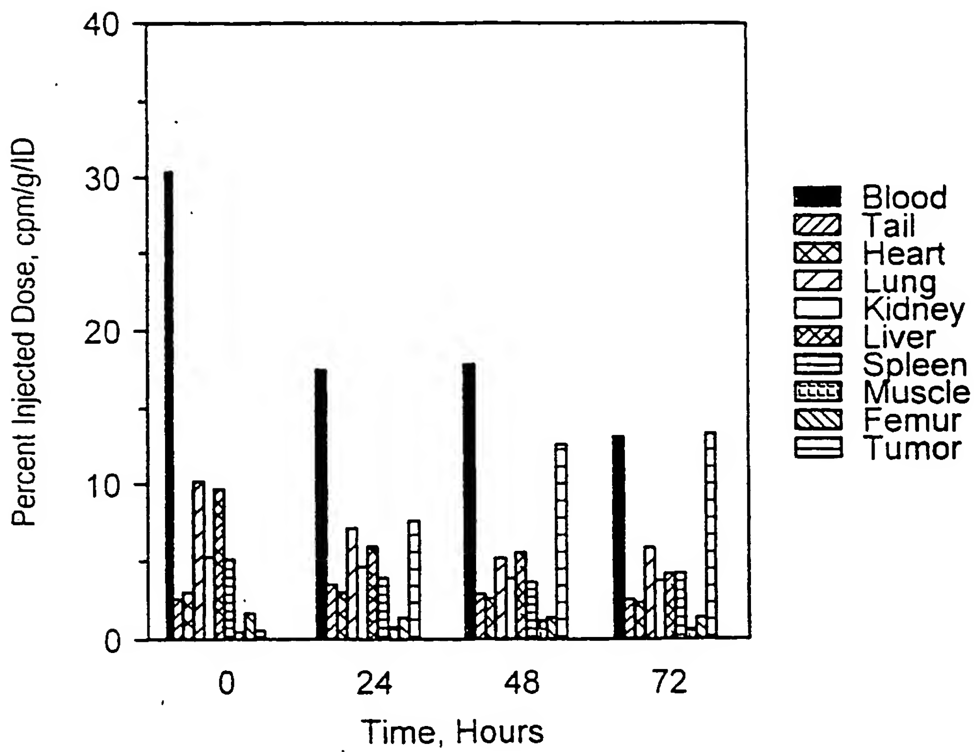


FIG. 26A

Binding of ^{90}Y-Labeled 2B8-MX-DTPA to CD20 Positive Human Cells

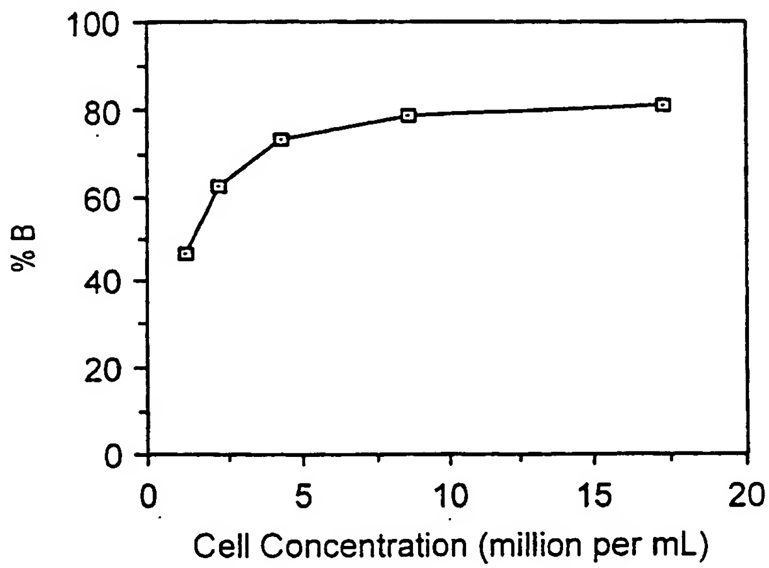


FIG. 26B

Binding of ^{90}Y-Labeled 2B8-MX-DTPA to CD20 Positive Human Cells

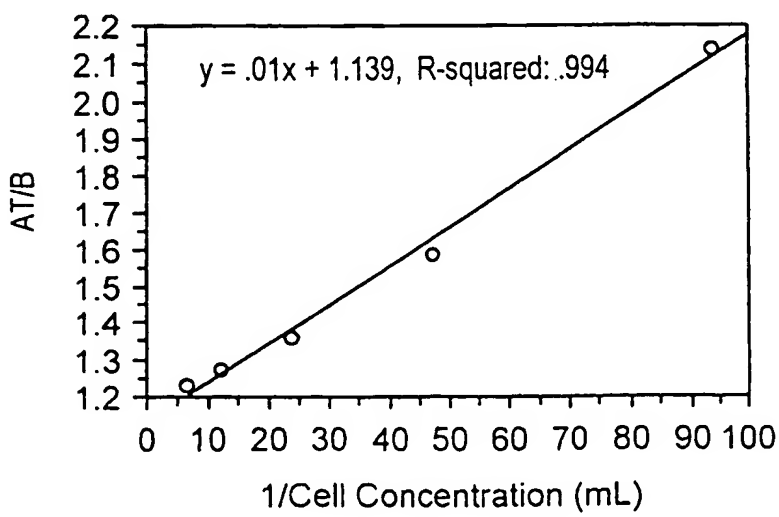


FIG. 27

In Vitro Stability of ^{90}Y-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin and DTPA

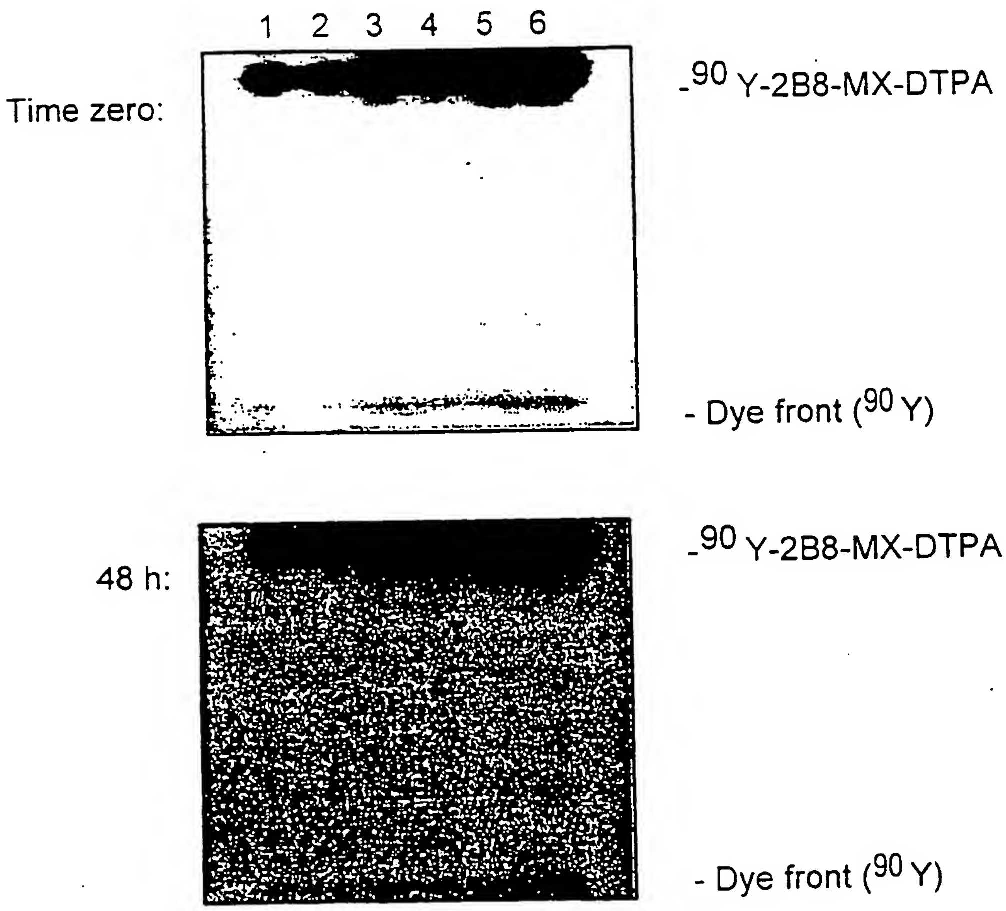


FIG. 28

In Vitro Stability of ^{90} Y-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin and DTPA

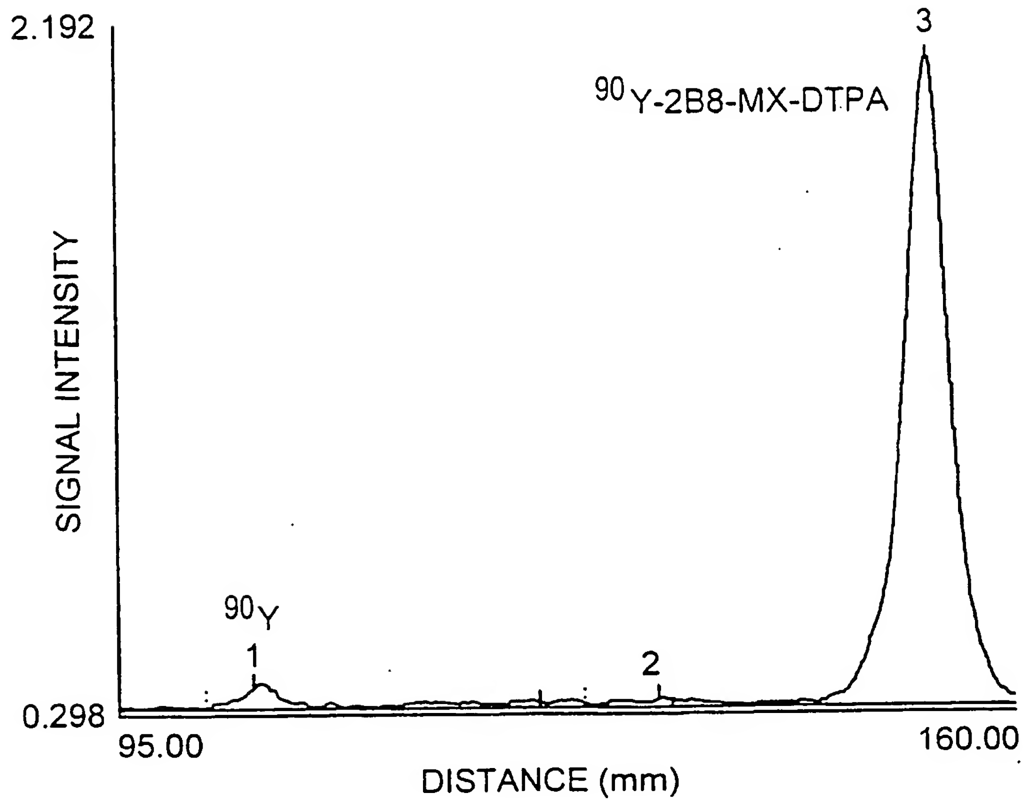


FIG. 29

In Vitro Stability of ^{90}Y-Labeled 2B8-MX-DTPA in PBS Containing Human Serum Albumin and DTPA

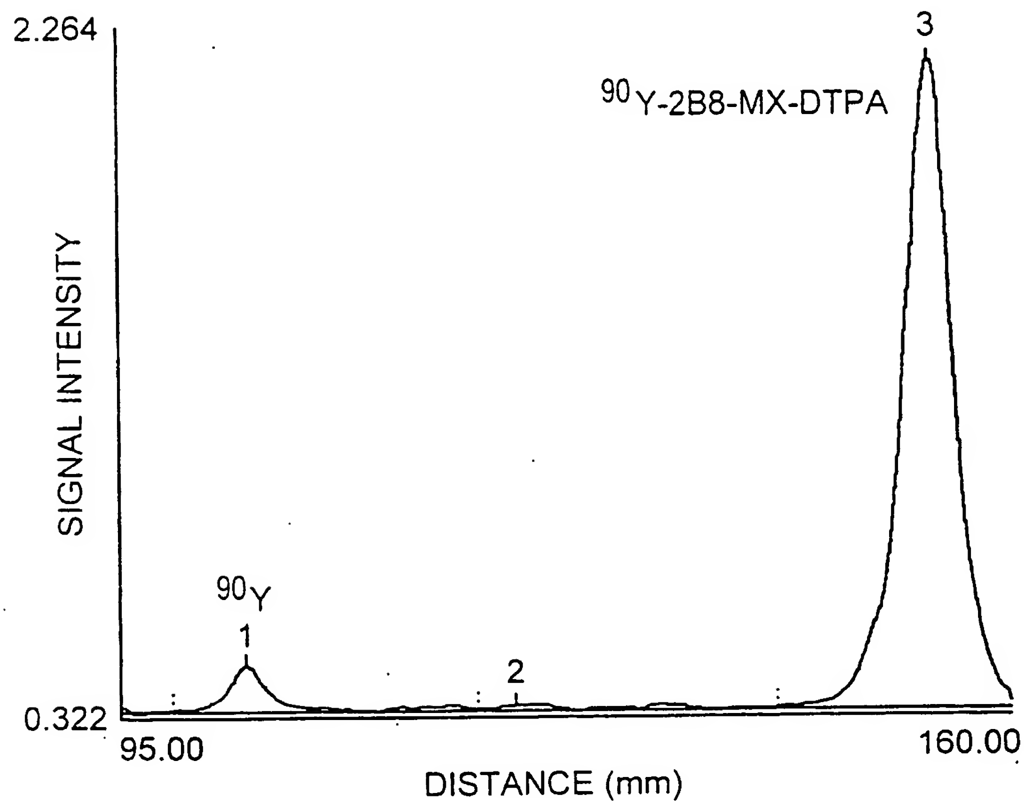


FIG. 30

In Vitro Stability of ^{90}Y-Labeled 2B8-MX-DTPA Incubated in Human Serum

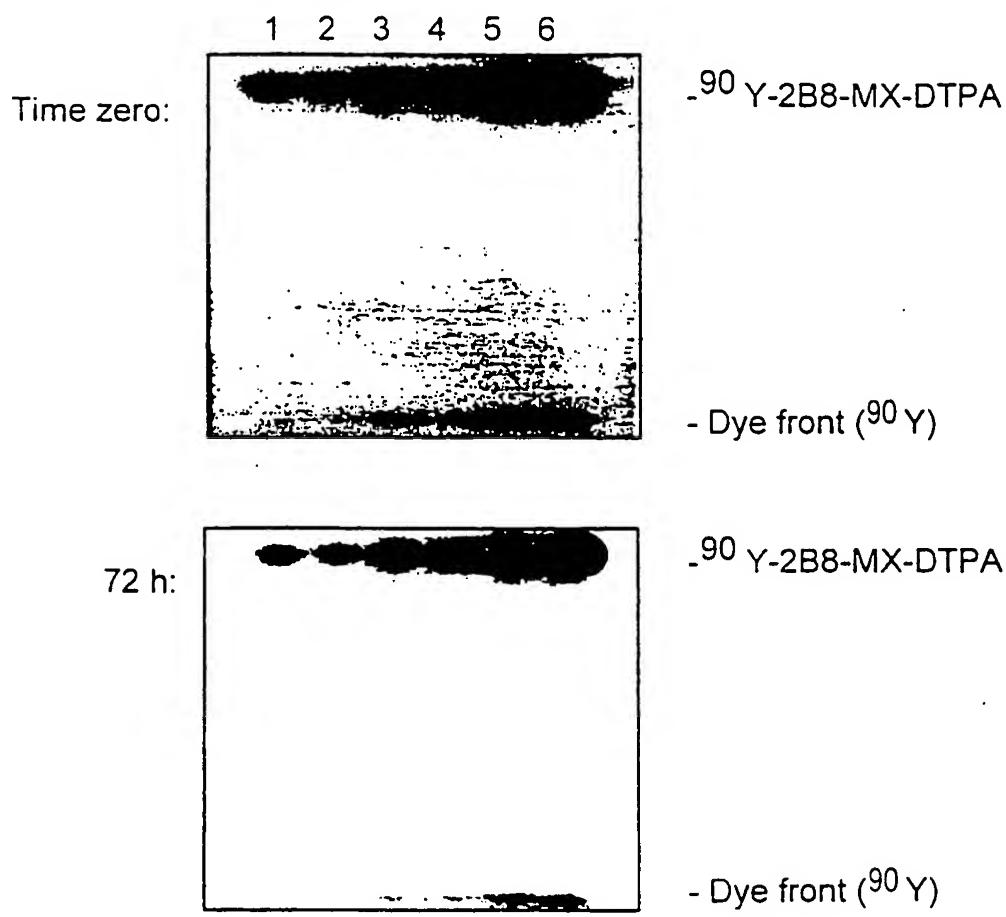


FIG. 31

In Vitro Stability of ^{90}Y-Labeled 2B8-MX-DTPA Incubated in Human Serum

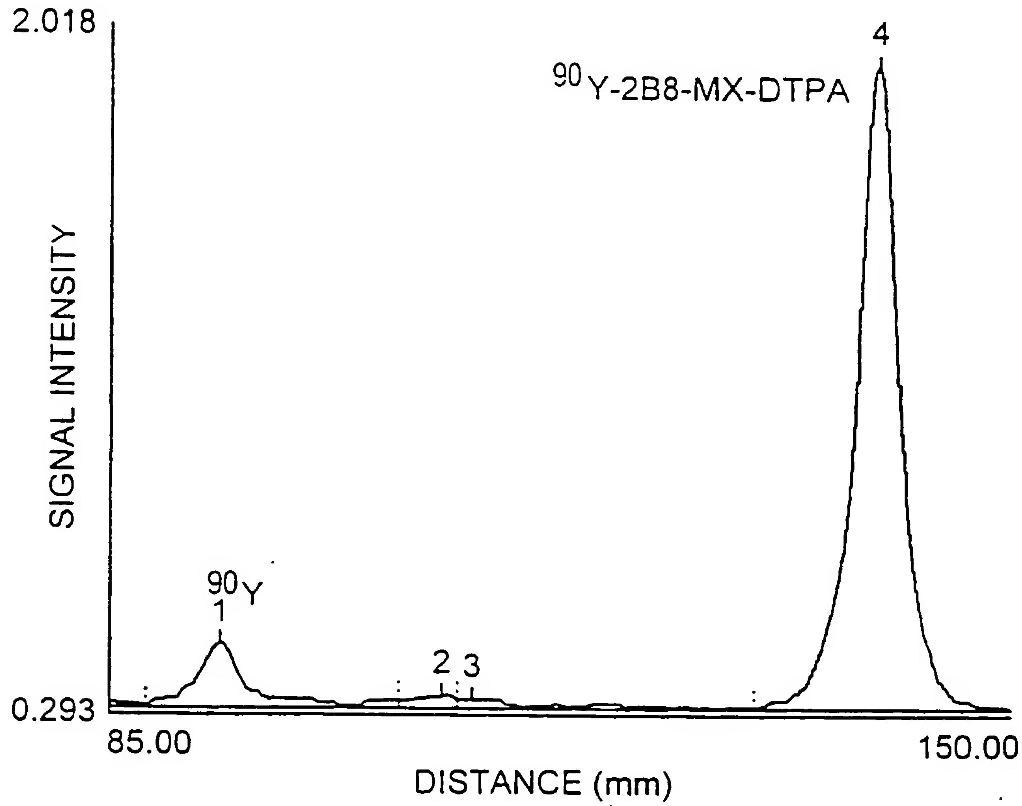


FIG. 32

In Vitro Stability of ^{90} Y-Labeled 2B8-MX-DTPA Incubated in Human Serum

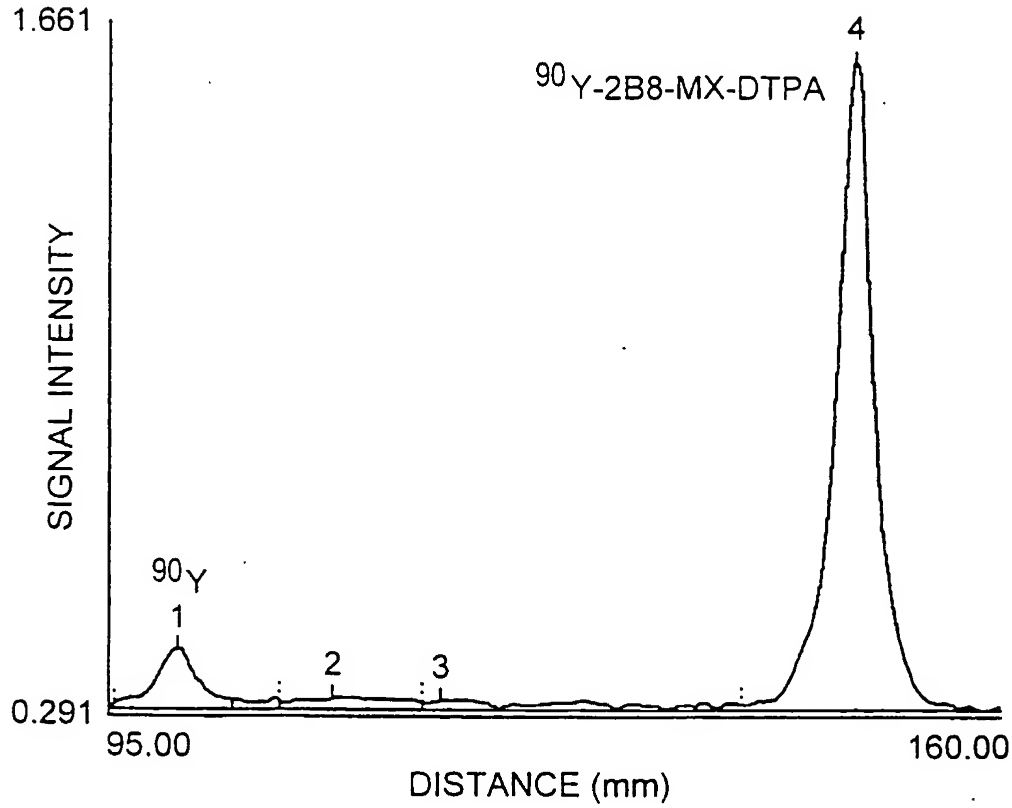


FIG. 33

Biodistribution of ^{90}Y-Labeled 2B8-MX-DTPA in Mice

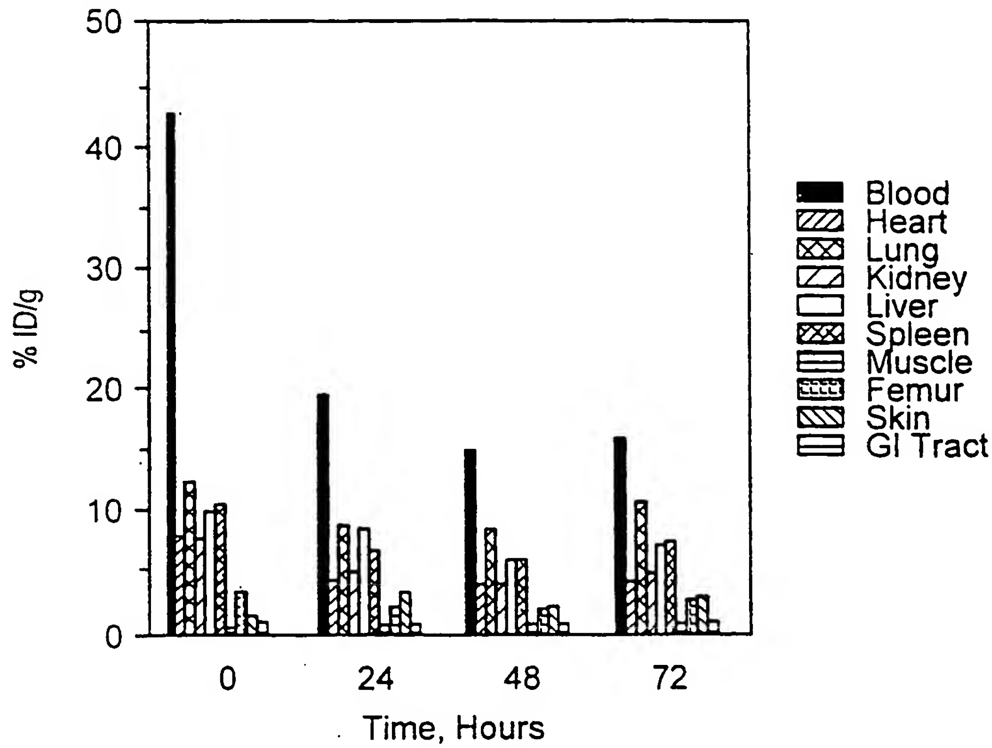


FIG. 34

Direct Binding of CHO-Derived 2B8 to CD20-Positive and CD20-Negative Human Cells

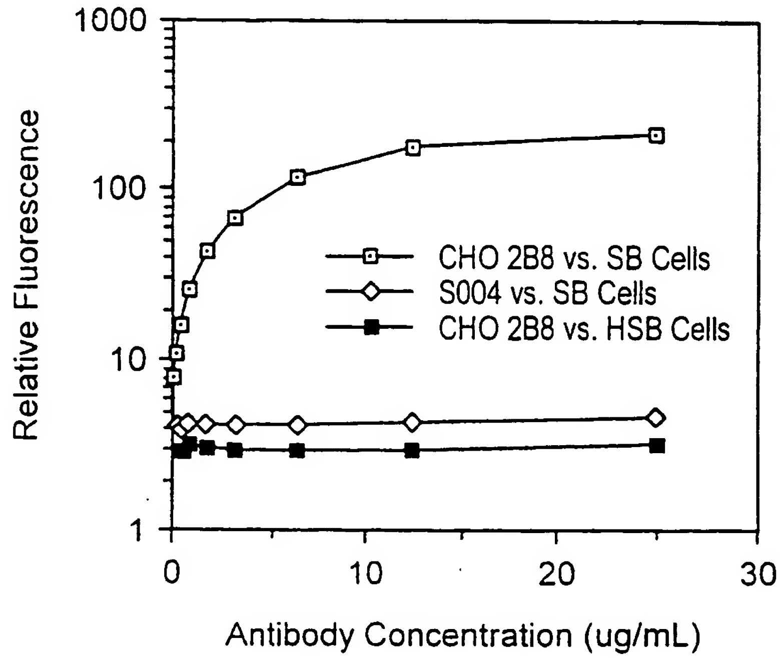


FIG. 35

Competitive Binding of CHO-Derived 2B8 to CD20-Positive Human Cells

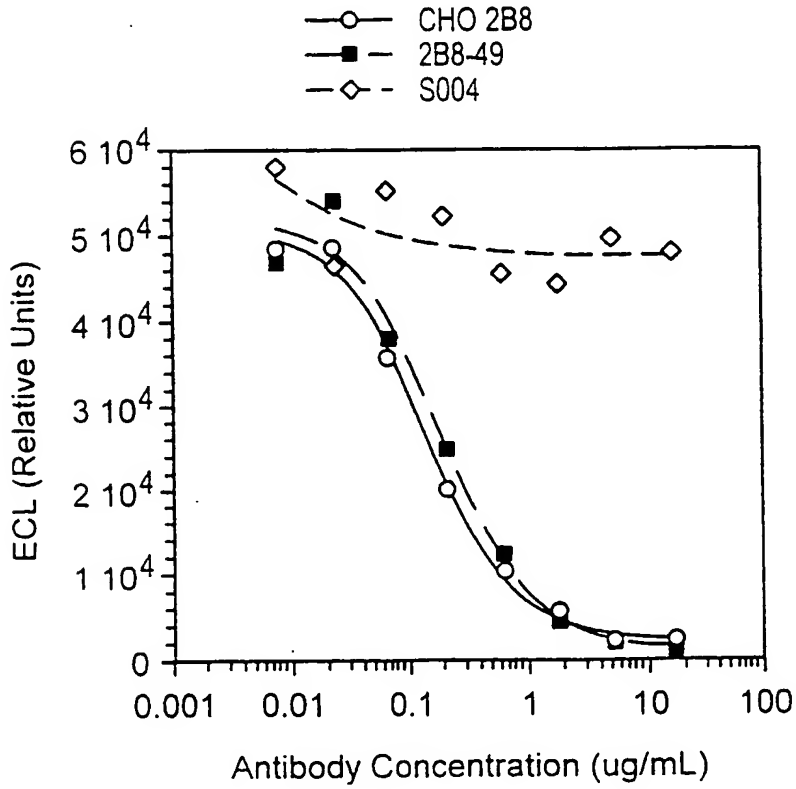


FIG. 36

Competitive Binding of CHO-Derived 2B8 and 2B8-MX-DTPA to CD20-Positive Human Cells

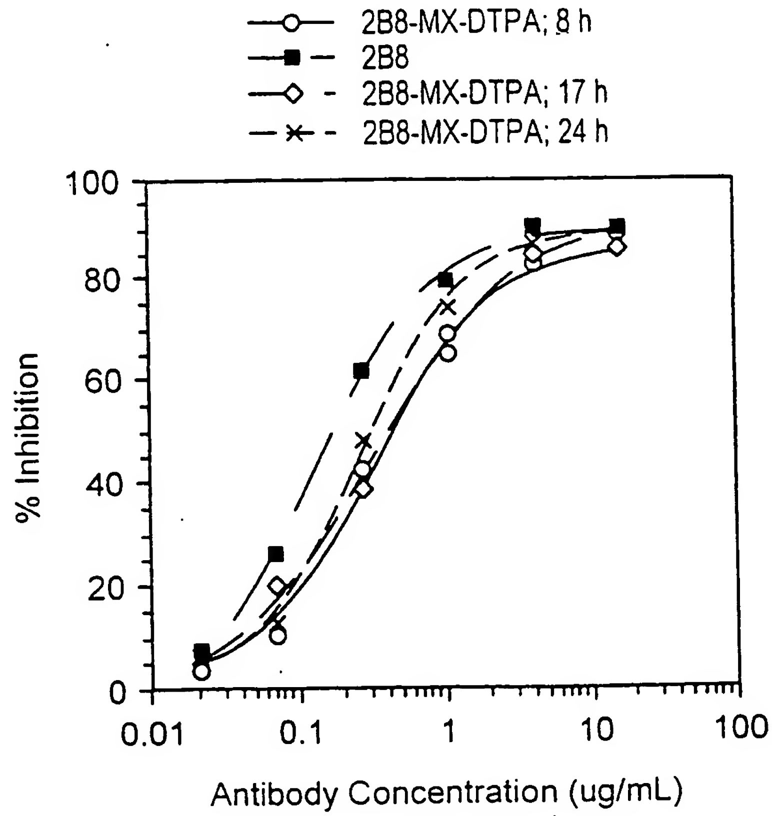


FIG. 37A

Binding of In2B8 Prepared From CHO 2B8-MX-DTPA to CD20-Positive Cells

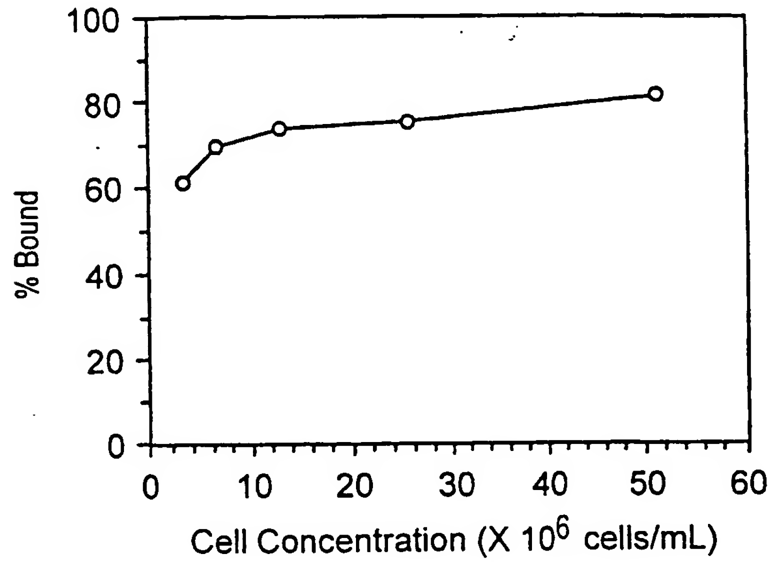


FIG. 37B

Binding ofIn2B8 Prepared From CHO 2B8-MX-DTPA to CD20-Positive Cells

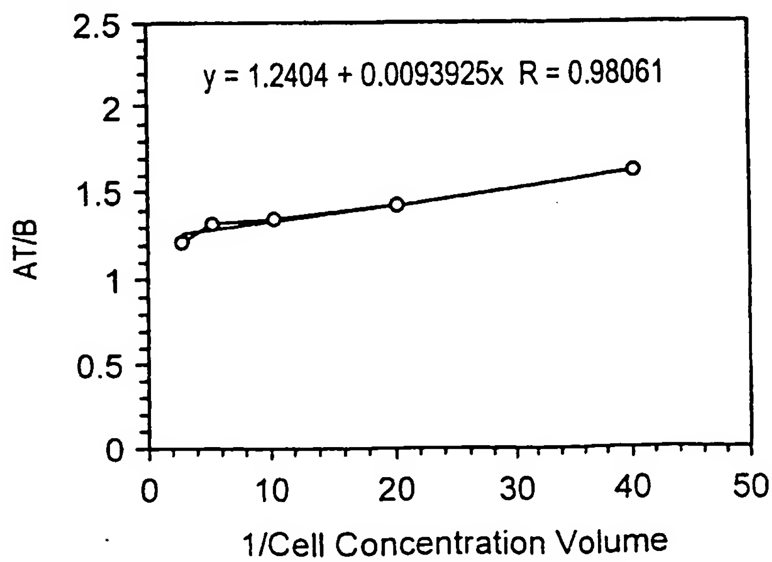


FIG. 38A

Binding of Y2B8 Prepared From CHO 2B8-MX-DTPA to CD20-Positive Cells

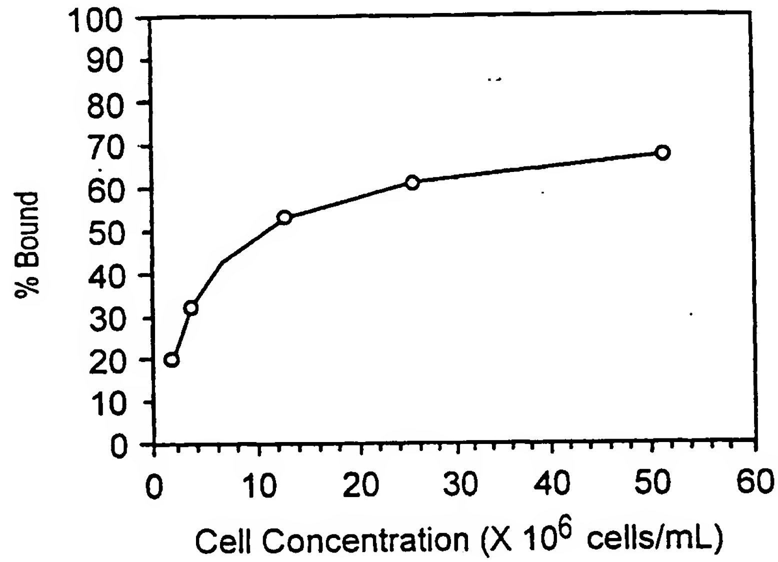
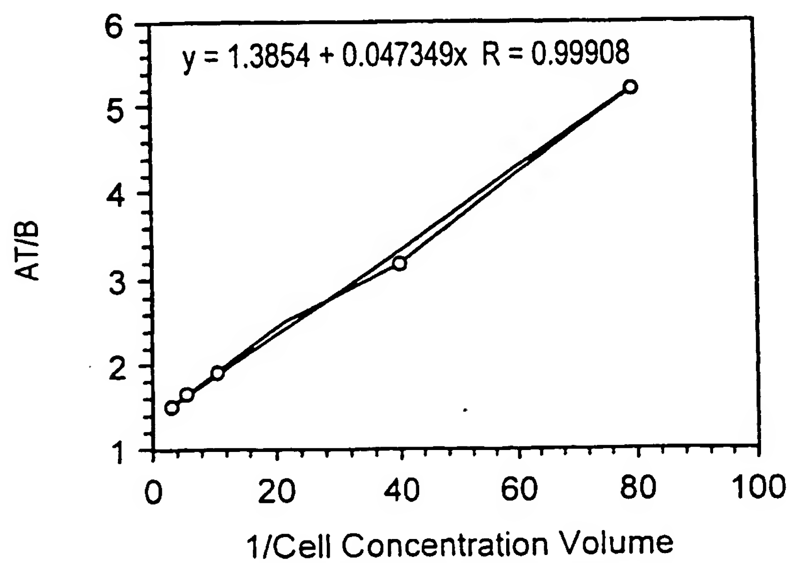


FIG. 38B

Binding of Y2B8 Prepared From CHO 2B8-MX-DTPA to CD20-Positive Cells



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